Regulation of $GABA_B$ receptor cell surface expression by degradation

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Learn from yesterday, live for today, hope for tomorrow.
The important thing is to not stop questioning."

Albert Einstein

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SUMMARY

γ-Aminobutyric acid (GABA) is the main inhibitory neurotransmitter in the brain and plays a major role in determining the routing or sculpting of neural activity, as well as limiting excessive neuronal firing, via the fast-acting ionotropic $GABA_A$ receptors and the slow-acting metabotropic $GABA_B$ receptors. Functional $GABA_B$ receptors are heterodimers comprising the subunits $GABA_{B1}$ and $GABA_{B2}$. Presynaptically, $GABA_B$ receptors inhibit voltage-gated Ca^{2+} channels and suppress neurotransmitter release whereas postsynaptically they induce slow inhibitory postsynaptic potentials by activation of Kir3-type K^+ channels. Dysfunction of $GABA_B$ receptors in the CNS is believed to be involved in various nervous system disorders such as addiction, anxiety, epilepsy, chronic pain and depression. Elucidation of the regulation of $GABA_B$ receptor signalling is essential for the understanding of the contribution of $GABA_B$ receptors to pathophysiological conditions. In this respect, the dynamic control of cell surface expression of the receptors is a main factor regulating $GABA_B$ receptor signalling. The aim of this thesis was to analyze to which extent degradation mechanisms regulate cell surface expression of $GABA_B$ receptors.

The research program was divided into three sub-projects. In project one we tested the hypothesis that the endoplasmic reticulum (ER)-associated protein degradation (ERAD) machinery may contribute to the regulation of $GABA_B$ receptor expression levels. We found that bidirectional modulation of proteasome activity increased and decreased, respectively, the expression level of $GABA_B$ receptors in cultured cortical neurons. Proteasomal degradation of $GABA_B$ receptors required K48-linked ubiquitination of two lysine residues in the C-terminal domain of $GABA_{B2}$. Blocking proteasome activity in neurons resulted in the accumulation of $GABA_B$ receptors in the endoplasmic reticulum, indicating that the degradation is mediated by the proteasome-dependent ERAD machinery. Pharmacological inhibition of ERAD or over-expression of a dominant-negative mutant form of VCP/p97, an essential protein of the ERAD complex, increased total as well as cell surface levels of $GABA_B$ receptors in neurons. In conclusion, our results indicate that the level of newly synthetized $GABA_B$ receptor in the ER available for cell surface trafficking is regulated by ERAD.

In project two; we searched for $GABA_B$ receptor-interacting proteins involved in proteasomal degradation and tested the hypothesis that proteasomal degradation might be regulated by neuronal activity. We found that the C-terminus of $GABA_{B2}$ interacts with Rpt6, one of the six AAA-ATPases of the 19S regulatory complex of the proteasome. Overexpression of Rpt6 in HEK293 cells strongly reduced the levels of co-expressed $GABA_{B2}$, whereas deletion of the C terminal domain of $GABA_{B2}$ or a synthetic peptide comprising the $GABA_{B2}$ interaction

site prevented the Rpt6-mediated down-regulation of $GABA_{B2}$. Blocking proteasomal activity in neurons increased the co-localization of $GABA_B$ receptors with Rpt6 and their expression level. A non-functional mutant of Rpt6 lacking its ATPase activity prevented degradation of $GABA_B$ receptors. Blocking ERAD activity decreased the interaction of Rpt6 with $GABA_B$ receptors, resulting in increased total as well as cell surface expression of the receptors. In addition, manipulation of neuronal activity, which had been shown to modulate proteasomal activity, affected cell surface expression of $GABA_B$ receptors and their interaction with Rpt6. In conclusion, these results suggest that the $GABA_{B2}$/Rpt6 interaction targets $GABA_B$ receptors to proteasomal degradation via the ERAD machinery in a neuronal activity-dependent manner and thereby regulates cell surface expression of $GABA_B$ receptors. This mechanism is expected to play an important role in homeostatic plasticity.

In the last project, we tested whether lysosomal degradation regulates cell surface expression of $GABA_B$ receptors and whether ubiquitination is a signal for directing the receptors to lysosomes. Inhibition of lysosomal activity in cortical neurons increased total and surface $GABA_B$ receptors, as well as K63-ubiquitination of the receptors. Mutational inactivation of four putative ubiquitination sites in $GABA_{B1}$ significantly diminished K63-linked ubiquitination of $GABA_{B1}$ and prevented an increase of their expression after pharmacological inhibition of lysosomal activity as well as by overexpressing a dominant-negative mutant of Rab7. This finding suggests that ubiquitination of $GABA_{B1}$ is a signal for lysosomal targeting. Furthermore, we demonstrate that lysosomal degradation of $GABA_B$ receptors is controlled by Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), which modulates K63-ubiquitination of $GABA_{B1}$. Overexcitation of neurons via prolonged exposure to glutamate, a condition that occurs in brain ischemia, rapidly down-regulates $GABA_B$ receptors through lysosomal degradation. Here we found that glutamate-induced down-regulation of $GABA_B$ receptors critically depends on CaMKII-mediated K63-linked ubiquitination of the receptors.

In conclusion, the present study revealed that proteasomal and lysosomal degradation pathways adjust cell surface expression of $GABA_B$ receptors according to the activity state of the neuron under physiological as well as pathological conditions.

ZUSAMMENFASSUNG

γ-Aminobuttersäure (GABA) ist der vorherrschende inhibitorische Neurotransmitter im Gehirn und ist ursächlich an der Regulation neuronaler Aktivität beteiligt. Die Wirkung von GABA wird über die ionotropen $GABA_A$-Rezeptoren und die metabotropen $GABA_B$-Rezeptoren vermittelt. $GABA_B$-Rezeptoren sind heterodimere Proteinkomplexe, die sich aus den Untereinheiten $GABA_{B1}$ und $GABA_{B2}$ zusammensetzen. Präsynaptische $GABA_B$-Rezeptoren inhibieren spannungsabhängige Ca^{2+}-Kanäle und verringern dadurch die Freisetzung von Neurotransmittern während postsynaptische $GABA_B$-Rezeptoren K^+-Kanäle aktivieren und so langsame, inhibitorische postsynaptische Potentiale induzieren. Es existieren Hinweise darauf, dass eine Fehlfunktion von $GABA_B$-Rezeptoren im ZNS an einer Vielzahl von neurologischen Erkrankungen beteiligt ist, wie z. B. Angststörungen, chronischen Schmerzen, Depressionen, Epilepsie und Sucht. Für ein Verständnis der Beteiligung von $GABA_B$-Rezeptoren an neurologischen Erkrankungen ist daher die Aufklärung seiner Regulationsmechanismen von wesentlicher Bedeutung. In dieser Hinsicht ist die dynamische Kontrolle der Zelloberflächen-Expression der $GABA_B$-Rezeptoren ein Hauptfaktor der die Signalstärke dieser Rezeptoren determiniert. Das Anliegen dieser Studie war aufzuklären in welchem Ausmass Degradations-Mechanismen die Expression der $GABA_B$-Rezeptoren an der Zelloberfläche regulieren.

Das Forschungsprogramm bestand aus drei Projekten. In Projekt 1 wurde die Hypothese getestet ob die „Endoplasmatische Retikulum (ER) assoziierte Protein Degradation“ (ERAD) an der Regulation der Zelloberflächen-Expression von $GABA_B$-Rezeptoren beteiligt ist. Die Untersuchungen ergaben, dass die Inhibition oder die Stimulierung der Proteasomen-Aktivität die $GABA_B$-Rezeptor-Expression in kultivierten Neuronen erhöhte bzw. verringerte. Für die proteasomale Degradation der $GABA_B$-Rezeptoren war eine K48-Polyubiquitinierung von zwei Lysinen in der C-terminalen Domäne von $GABA_{B2}$ notwendig. Die Blockierung der proteasomalen Aktivität führte zu einer Akkumulierung der $GABA_B$-Rezeptoren im ER. Dies deutet darauf hin, dass die Rezeptoren durch die Proteasomen-abhängige ERAD-Maschinerie abgebaut werden. Die pharmakologische Inhibition von ERAD oder die Überexpression einer funktionell inaktiven Mutante von VCP/p97, einer essenziellen Komponente des ERAD-Komplexes, erhöhte die totale und Zelloberflächen-Expression der $GABA_B$-Rezeptoren in Neuronen. Die Resultate dieser Untersuchungen zeigen, dass die Menge an neu gebildeten $GABA_B$-Rezeptoren im ER, welche an die Zelloberfläche exportiert werden können, durch ERDA reguliert wird.

In Projekt 2 wurde nach $GABA_B$-Rezeptor interagierenden Proteinen gesucht, die an der proteasomalen Degradation der Rezeptoren beteiligt sind und der Frage nachgegangen ob die proteasomale Degradation der Rezeptoren durch die neuronale Aktivität reguliert wird. Die Untersuchungen ergaben, dass der C-Terminus von $GABA_{B2}$ mit Rpt6 interagiert, einer der sechs AAA-ATPasen der 19S regulatorischen Untereinheit des Proteasoms. Überexpression

von Rpt6 in HEK203 Zellen führte zu einer starken Abnahme der Menge an koexprimiertem $GABA_{B2}$. Die Deletion der C-terminalen Domäne von $GABA_{B2}$ oder ein synthetisches Peptid, welches die $GABA_{B2}$/Rpt6-Bindungsstelle repräsentiert, verhinderte die Rpt6 vermittelte Herunterregulierung von $GABA_{B2}$. Die Blockierung der proteasomalen Aktivität in Neuronen verstärkte die Kolokalisation der $GABA_B$-Rezeptoren mit Rpt6 sowie das Expressionsniveau der Rezeptoren. Weiterhin konnte gezeigt werden, dass eine funktionell inaktive Rpt6 Mutante den Abbau der Rezeptoren verhinderte und die Inhibition von ERAD zu einer verminderten $GABA_B$-Rezeptor/Rpt6-Interaktion sowie zu einem erhöhten Expressionsniveau totaler und Zelloberflächen-Rezeptoren führte. Die Manipulation der neuronalen Erregung, welche wiederum die Aktivität der Proteasomen moduliert, beeinflusste die $GABA_B$-Rezeptor/Rpt6-Interaktion und als Konsequenz die Zelloberflächen-Expression der Rezeptoren. Diese Ergebnisse zeigen, dass die Interaktion von Rpt6 mit $GABA_{B2}$ essentiell ist für den proteasomalen Abbau der $GABA_B$-Rezeptoren durch die ERAD-Maschinerie. Das Ausmass des proteasomale Abbaus der Rezeptoren wird durch den Aktivitätszustand des Neurons bestimmt und dadurch die Zelloberflächenexpression der $GABA_B$-Rezeptoren reguliert. Dieser Mechanismus spielt höchst wahrscheinlich eine wichtige Rolle in der homeostatischen Plastizität der Neurone.

Im letzten Projekt (Projekt 3) wurde der Frage nachgegangen inwieweit die lysosomale Degradation der $GABA_B$-Rezeptoren deren Zelloberflächen-Expression reguliert und ob Ubiquitiniertung der Rezeptoren als lysosomales Sortierungssignal dient. Inhibition der lysosomalen Aktivität in Neuronen erhöhte die Expression sowohl der totalen als auch der Zelloberflächen-Rezeptoren sowie deren K63-Ubiquitinierung. Die Inaktivierung von vier potenziellen Ubiquitinierungsstellen in $GABA_{B1}$ durch gerichtete Mutagenese verminderte die K63-Ubiquitinierung von $GABA_{B1}$ beträchtlich und verhinderte die erhöhte $GABA_B$-Rezeptor-Expression nach Blockierung der lysosomalen Aktivität. Dieses Resultat weist darauf hin, dass Ubiquitinierung von $GABA_{B1}$ ein Signal für den lysosomalen Abbau der Rezeptoren ist. Weiterhin ergaben die Untersuchungen, dass die lysosomale Degradation der $GABA_B$-Rezeptoren durch die Ca^{2+}/Calmodulin-abhängige Proteinkinase II (CaMKII) reguliert wird via Modulation der K63-Ubiquitinierung von $GABA_{B1}$. Übererregung von Neuronen durch anhaltende Applikation von Glutamat, eine Situation die bei der zerebralen Ischämie auftritt, führt zu einer schnellen Herunterregulierung der $GABA_B$-Rezeptoren durch verstärkten lysosomalen Abbau. Unsere Untersuchungen ergaben, dass die Glutamat-induzierte Herunterregulierung der $GABA_B$-Rezeptoren von der CaMKII-vermittelten K63-Ubiquitinierung der Rezeptoren abhängt.

Die Ergebnisse dieser Arbeit ergaben, dass sowohl der proteasomale als auch der lysosomal Abbauweg die Zelloberflächen-Expression der $GABA_B$-Rezeptoren in Abhängigkeit von der neuronal Aktivität unter physiologischen wie auch unter pathologischen Bedingungen reguliert.

General Introduction

GABA RECEPTORS

γ-Aminobutyric acid (GABA) is the most important inhibitory neurotransmitter in the central nervous system. GABA mediates its action via the ionotropic $GABA_A$ receptors and the metabotropic $GABA_B$ receptors (Bettler et al 2004a). Upon binding of GABA, $GABA_A$ receptors mediate fast synaptic inhibition by activation of an intrinsic chloride channel, whereas $GABA_B$ receptors mediate slow and prolonged neuronal inhibition by indirectly affecting downstream effector systems via $G_{i/o}$ proteins. $GABA_B$ receptors are distributed throughout the central nervous system and are expressed both in inhibitory and in excitatory neurons (Fritschy et al 1999, Fritschy et al 2004). $GABA_B$ receptors are involved in the regulation of virtually all main brain functions like synaptic plasticity (Pinard et al 2010), neuronal network activity (Craig & McBain 2014, Kohl & Paulsen 2010) and neuronal development (Gaiarsa et al 2011, Gaiarsa & Porcher 2013). Accordingly, $GABA_B$ receptors have been implicated in a variety of neurological disorders including anxiety, chronic pain, depression, drug addiction, epilepsy and spasticity (Benarroch 2012, Bettler et al 2004a, Bowery et al 2004, Bowery 2006, Bowery et al 2002, Cryan & Kaupmann 2005, Kumar et al 2013, Mombereau et al 2005).

STRUCTURE OF $GABA_B$ RECEPTORS

Functional $GABA_B$ receptors are heterodimers comprising the subunits $GABA_{B1}$ and $GABA_{B2}$. Both subunits are integral membrane proteins containing seven transmembrane domains (TM), a large extracellular domain constituting a “Venus fly-trap” domain and a large C-terminal domain with a coiled-coil structure that serves as a binding domain for several interacting proteins (Fig.1) (Bettler et al 2004a). Assembly of $GABA_B$ subunits takes place in the endoplasmatic reticulum (ER) is required for leaving the ER and trafficking to the plasma membrane. $GABA_{B1}$ contains an ER retention signal (RSRR) near the coiled-coil domain, which prevents ER exit of unassembled $GABA_{B1}$ (Couve et al 1998, Margeta-Mitrovic et al 2000, Pagano et al 2001a) by binding to the coat protein complex I (COPI) (Brock et al 2005). Assembly with $GABA_{B2}$ masks the $GABA_{B1}$ RSRR motif and permits forward trafficking of the heterodimeric receptor (Pagano et al 2001a).

Within the receptor complex, both subunits fullfil distinct functions: $GABA_{B1}$ harbors the binding site for orthosteric ligands in the extracellular “Venus fly-trap” domain, while the Venus fly-trap domain present in $GABA_{B2}$ is constantly in a closed conformation preventing ligand binding (Galvez et al 1999, Geng et al 2013). $GABA_{B2}$ contains a binding site for

allosteric modulators within the transmembrane domain (Binet et al 2004) and recruits the $G_{i/o}$ proteins (Duthey et al 2002, Galvez et al 2001, Havlickova et al 2002, Robbins et al 2001) .

$GABA_{B1}$ exists in two main variants, $GABA_{B1a}$ and $GABAB_{1b}$ (Fig. 1), which is generated by alternative promoter usage (Steiger et al 2004). Accordingly, they constitute the two $GABA_B$ receptor subtypes ($GABA_{B1a,2}$ and $GABA_{B1b,2}$), which are abundantly expressed throughout the brain with distinct but overlapping expression patterns in the developing and adult brain (Benke et al 1999, Fritschy et al 1999, Fritschy et al 2004, Vigot et al 2006) . $GABA_{B1a}$ and $GABA_{B1b}$ solely differ in their N-terminus by the presence of a pair of sushi domains (protein-protein interaction domains) in $GABA_{B1a}$, which have been shown to be involved in axonal targeting of $GABA_{B1a,2}$ receptors (Biermann et al 2010)and in increased cell surface stability of the receptors (Hannan et al 2012).

EFFECTOR SYSTEMS OF $GABA_B$ RECEPTOR

$GABA_B$ receptors recruit and activate $G_{i/o}$ type G proteins (Campbell et al 1993, Duthey et al 2002, Galvez et al 2001, Havlickova et al 2002, Robbins et al 2001). Activation of $GABA_B$ receptors mainly triggers adenylyl cyclase-mediated effects, inhibits ion channel-mediated transmitter release and regulates neuronal excitability (Gage 1992, Hill 1985, Hill et al 1984) (Fig.1).

At presynaptic sites, activation of $GABA_B$ receptors inhibits neurotransmitter release by reducing the activity of P/Q- and N-type voltage-gated Ca^{2+} channels via $G\beta\gamma$ (Fox et al 1978, Pierau & Zimmermann 1973). In addition, $G\alpha_{i/o}$ of the $GABA_B$ receptor-activated G protein contributes to the inhibition of transmitter release by increasing the energy barrier for vesicle fusion (Rost et al 2011). Under conditions of sustained neuronal activity, $GABA_B$ receptor activated $G\alpha_{i/o}$ retards recruitment of synaptic vesicles due to decreased cAMP levels (Sakaba & Neher 2003).

At postsynaptic sites, $GABA_B$ receptor-activated $G\beta\gamma$ triggers the opening of inwardly rectifying K^+ channels (Kir3 type), thereby inducing slow inhibitory postsynaptic currents (Bormann 1988, Dutar & Nicoll 1988, Luscher et al 1997, Wagner & Dekin 1993). $GABA_B$ receptor-activated $G\alpha_{i/o}$ inhibits adenylate cyclases, resulting in decreased intracellular cAMP levels and consequently diminished cAMP-dependent protein kinase (PKA) activity (Karbon et al 1984, Watling & Bristow 1986, Wojcik & Neff 1984). This has two main $GABA_B$ receptor-mediated effects. First, the reduced PKA activity releases a tonic block from two pore K^+ channels (TREK1/2), which induces an inhibitory postsynaptic current (Deng et al

2009, Sandoz et al 2012). Second, Ca^{2+} signals mediated by NMDA receptors, which are controlled by PKA activity, are inhibited by activation of $GABA_B$ receptors (Chalifoux & Carter 2010). This is suggested to influence NMDA receptor-dependent synaptic plasticity.

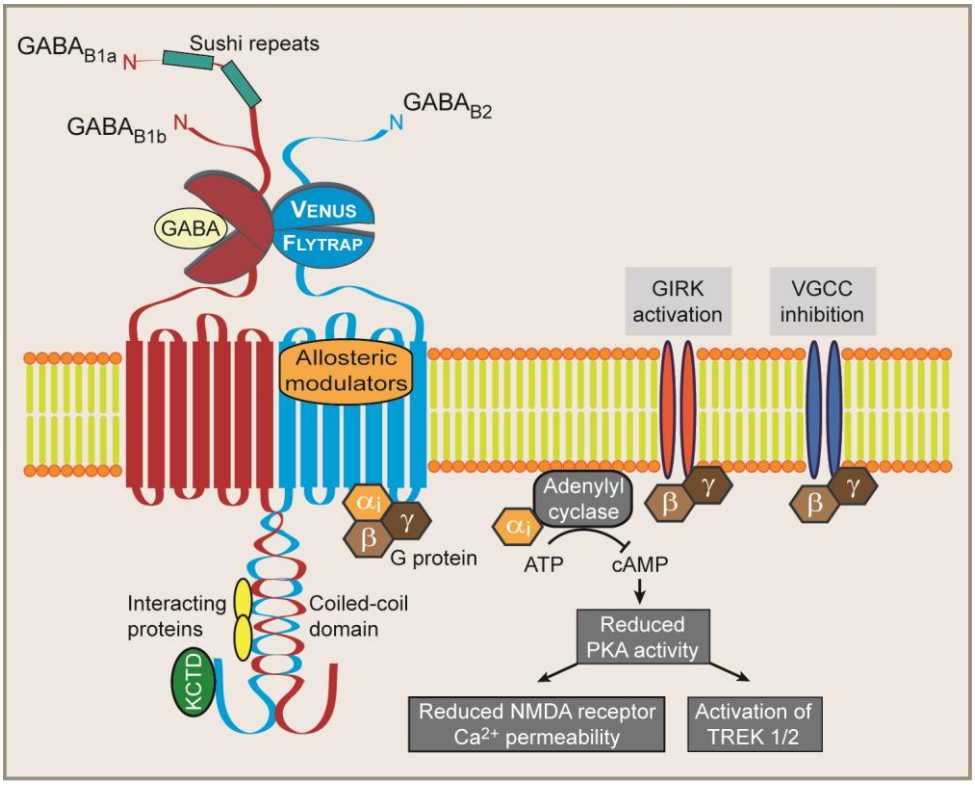

Figure 1: Structure and effector systems of $GABA_B$ receptors. The $GABA_B$ receptor is a heterodimer comprising the two subunits $GABA_{B1}$ and $GABA_{B2}$. $GABA_{B1}$ exists in two variants, $GABA_{B1a}$ and $GABA_{B1b}$. They differ only in the N-terminus by the presence of two sushi repeates in $GABA_{B1a}$. The two subunits subserve distinct functions. Whereas $GABA_{B1}$ is responsible for binding of orthosteric ligands, $GABA_{B2}$ contains a binding site of allsteric modulators and recruits G proteins of the $G_{i/o}$ type. The α subunit of the activated G protein inhibits the adenylate cyclase (AC), which leads to diminished cAMP levels and reduced PKA activity. This in turn activates TREK1/2 K^+ channels and inhibits Ca^{2+} signals mediated by NMDA receptors. Depending on the localization, the βγ dimer of the activated G protein regulates distinct effector system. At postsynaptic sites, Gβγ triggers the opening of inwardly rectifying K^+ channels (Kir 3) resulting in the hyperpolarization of the membrane. Presynaptic $GABA_B$ receptors repress the opening of Ca^{2+} channels and thereby the transmitter.

$GABA_B$ RECEPTOR TRAFFICKING

The number of $GABA_B$ receptors available for signaling is determined by various mechanisms including the amount of receptor synthesis, maturation and assembly of the receptors, their kinetic of forward transport from the ER to the plasma membrane, their rates of endocytosis from and recycling to the cell surface as well as their rate of degradation. All these events control the life time and presence of the receptors at the cell surface and thus determine to a large extent their maximal possible signaling strength (Fig. 2).

To date little is known about the synthesis, folding, assembly and forward trafficking of $GABA_B$ receptors to the cell surface. It is however clear that newly synthesized $GABA_B$ receptors need to be assembled within the ER into heterodimers for forward trafficking to the cell surface. This is due to the presence of an ER retention signal located within the C-terminal domain of $GABA_{B1}$ (Calver et al 2001, Margeta-Mitrovic et al 2000, Pagano et al 2001a). Interaction of COPI (a constituent of the machimery for retrograde protein transport) with the ER retention signal of $GABA_{B1}$ redirect unassembled escaped subunits from the *cis*-Golgi compartment back to the ER (Brock et al 2005). Efficient forward trafficking of $GABA_{B1}$ (in which the ER-retention signal was inactivated to permit ER exit) from the *trans*-Golgi network to the plasma membrane appears to require a LL-motif located within the coiled-coil domain of $GABA_{B1}$ (Restituito et al 2005). It is however unclear whether the LL-motif is involed in forward trafficking of the heterodimeric receptor complex since it is most likely masked by the assembly with $GABA_{B2}$.

Once the receptors reach the plasma membrane their residence time is determined by its rates of endocytosis, recycling and degradation. It is now well established that $GABA_B$ receptors constitutively internalize with a high rate via the classical clathrin and dynamin-dependent pathway (Grampp et al 2007b, Laffray et al 2007, Vargas et al 2008). Cell surface labeling experiments and life cell imaging suggest that $GABA_B$ receptors in cultured neurons are rapidly endocytosed (50% of cell surface receptors are internalized within 2-10 min) (Hannan et al 2011b, Maier et al 2010b, Wilkins et al 2008) . Endocytosis of $GABA_B$ receptors involve the interaction with the AP2 (adapter protein 2) complex, which recruit membrane proteins to clathrin-coated pits (Grampp et al 2008b, Grampp et al 2007b, Vargas et al 2008). Internalized $GABA_B$ receptors are sorted from early endosomes either to Rab4 and Rab11-positive recycling endosomes for reinsertion into the plasma membrane or to lysosomes for degradation (Grampp et al 2008b, Hannan et al 2011b, Laffray et al 2007, Vargas et al 2008). In addition, activation of $GABA_B$ receptors with baclofen has been shown to accelerate

recycling of receptors and blocking recycling diverts the receptors to lysosomes for degradation (Grampp et al 2008).

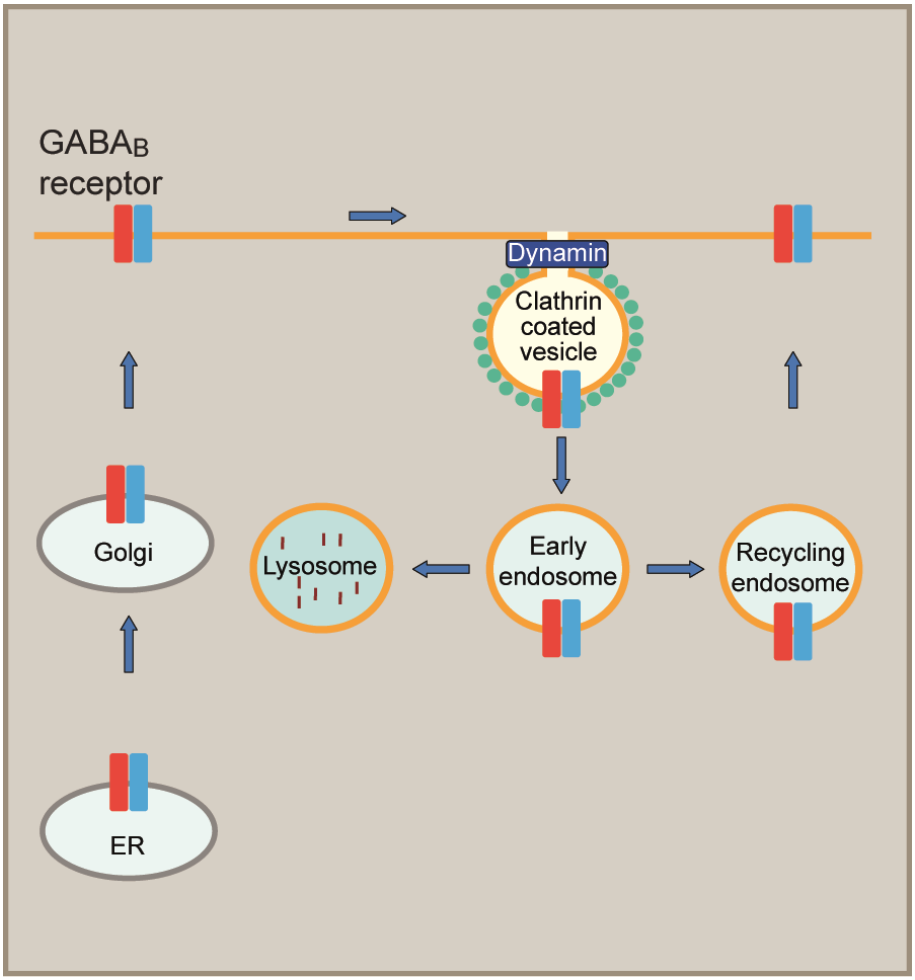

Figure 2. Pathways of $GABA_B$ receptor trafficking. $GABA_B$ receptors are synthesized and inserted into the ER membrane where the subunits assemble into the heterodimeric receptor (red and blue boxes). They are then transported via the Golgi apparatus to the plasma membrane. $GABA_B$ receptors at the plasma membrane are constitutively endocytosed via the clathrin and dynamin-dependent mechanism. Internalized $GABA_B$ receptors are sorted from early endosomes either to recycling endosomes for reinsertion into the plasma membrane or to lysosomes for degradation.

DEGRADATION OF $GABA_B$ RECEPTORS

There is currently only limited information available on the degradation pathways of $GABA_B$ receptors. Based on their intracellular accumulation upon inhibition of lysosomal protein degradation (Grampp et al 2008a, Grampp et al 2007b, Kantamneni et al 2008a) and their colocalization with lysosomal marker proteins (Grampp et al 2008a, Hannan et al 2011a) there is no doubt that $GABA_B$ receptors are eventually degraded in lysosomes. There is first indications that the ESCRT (endosomal sorting complex required for transport) machinery, which sorts ubiquitinated proteins to lysosomes, is involved in targeting $GABA_B$ receptors to lysosomes. Knockdown of the ESCRT protein TSG101 (tumor susceptibility gene 101) prevented degradation of $GABA_B$ receptors (Kantamneni et al 2008a).

Lysosomal degradation and recycling of $GABA_B$ receptors need to be tightly regulated to ensure the correct number of receptors at the cell surface. There is evidence that both mechanisms are interconnected and influence each other. For instance, inhibition of receptor recycling redirected the receptors to lysosomes for degradation (Grampp et al 2008). Furthermore, sustained activation of glutamate receptors (a condition that occurs in brain ischemia) rapidly down-regulates $GABA_B$ receptors by shifting the recycling/degradation balance toward lysosomal degradation (Guetg et al 2010, Kantamneni et al 2014, Maier et al 2010b, Terunuma et al 2010). However, the precise signals regulating $GABA_B$ receptor degradation as well as the contribution of alternative degradation pathways such as proteasomal degradation are unknown.

MECHANISMS OF PROTEIN DEGRADATION

The precise regulation of degradation is fundamental for almost all cellular processes. Accordingly, eukaryotic cells have evolved complex machineries for protein degradation (Ciechanover 2006). The major proteases in eukaryotic cells are confined to specialized organelles (lysosomes) and protein complexes (proteasomes) (Fig. 3). The vast majority of plasma membrane proteins are degraded in lysosomes, which are heterogeneously shaped vacuoles containing lysosomal hydrolases for protein degradation (Saftig & Klumperman 2009). In contrast, most soluble and intracellular membrane proteins are degraded by proteasomes (Goldberg 2007, Hicke 2001).

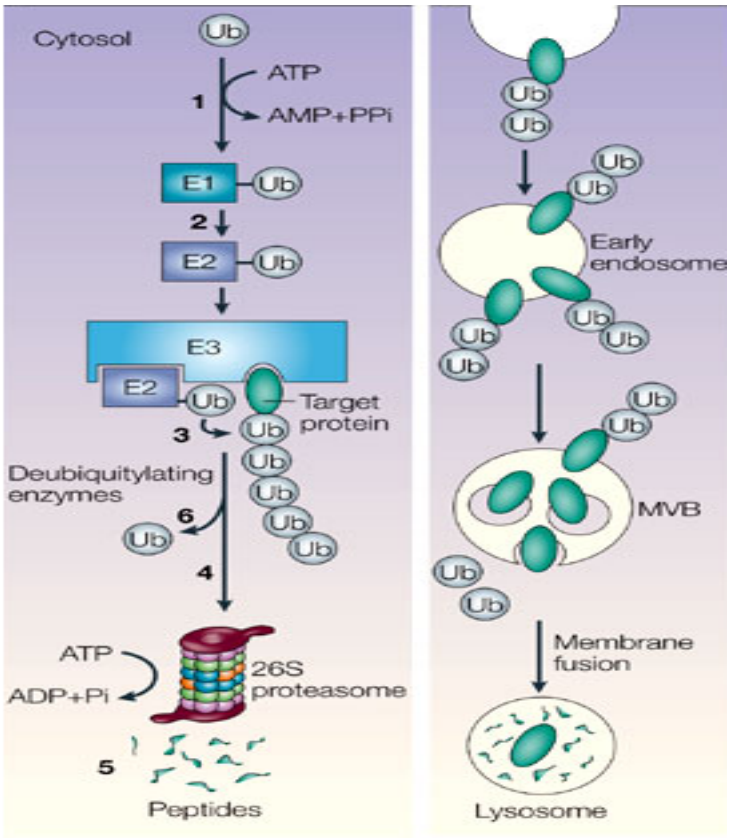

Figure 3. Mechanisms of protein degradation. Proteins are degraded either by proteasomes (left) or lysosomes (right). Sorting of proteins from early endosomes via multivesicular bodies (MVB) to lysosomes involves ubiquitination of the target protein with either a single ubiquitin to one or multiple sites of a substrate protein (mono or multiple mono-ubiquitination) or with K63-linked polyubiquitin. K48-linked polyubiquitination is usually required for proteasomal degradation. The figure was taken from (Ciechanover 2005)

Ubiquitin tags proteins for lysosomal and proteasomal degradation

Ubiquitin is a 76-amino acid residue protein with a size of 8 kDa, which serves not only as a signal for proteasomal degradation but is also a sorting signal for the regulation of protein transport between membrane compartments. In a process called ubiquitination, ubiquitin is covalently attached to lysine (K) residues of the target protein (Hershko & Ciechanover 1998). The conjugation of ubiquitin to the target protein requires three enzymes (Fig. 4): an ubiquitin-activating enzyme (E1), an ubiquitin-conjugation enzyme (E2) and an ubiquitin ligase (E3). Ubiquitin is first activated by the E1 enzyme in an ATP-dependent reaction by formation of a thiolester bond with this enzyme and is then transferred to the E2 enzyme. In the final step the E3 enzyme links the C-terminal glycine of ubiquitin to a lysine side chain of the substrate protein by forming a stable isopeptide bond (Hershko & Ciechanover 1998).

There are several ways how proteins can be modified by ubiquitin. The simplest one is monoubiquitination, where a single ubiquitin is attached to a target protein (Hicke et al., 2004). Another modification is multiple-ubiquitination, where multiple single ubiquitin molecules are attached to several lysine residues in the target protein. Finally, polyubiquitin chains can be attached to the target protein. These polyubiquitin chains are formed by the conjugation of the ubiquitin C-terminus to one of the seven lysine residues (K6, K11, K27, K29, K33, K48 and K63) of another ubiquitin (Pickart and Fushman, 2004; Ikeda and Dikic, 2008) (Fig. 3). Polyubiquitin chains linked via K48 and via K63 both function as a signal for degradation but via different routes. K48-linked chains mark proteins for proteasomal degradation (Chau et al 1989, Hershko & Ciechanover 1992, Weissman 2001), whereas K63-linked chains serve as a signal for sorting internalized plasma membrane proteins to lysosomes via the multivesicular body (MVB) pathway (Duncan et al 2006, Huang et al 2006, Lauwers et al 2009, MacGurn et al 2012).

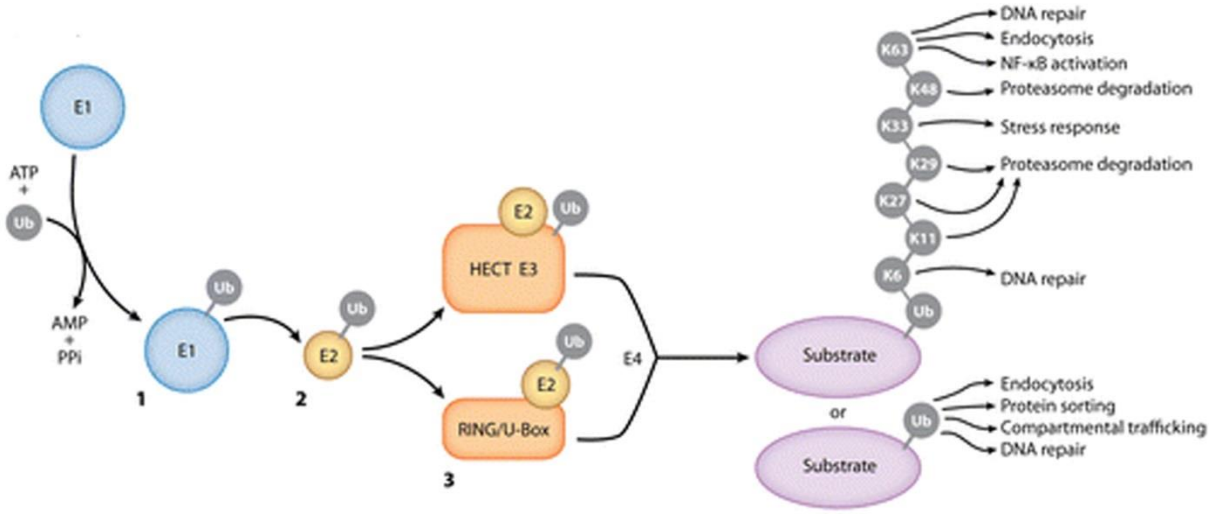

Figure 4: The ubiquitin pathway. Ubiquitination comprises three subsequential enzymatic reactions. In step 1, free ubiquitin is activated by an ubiquitin-activating enzyme (E1) via an ATP-dependent reaction. In step 2, the activated ubiquitin is transferred to a cysteine of an ubiquitin-conjugating enzyme (E2) via a thioester linkage. In step 3, an ubiquitin ligase (E3) couples the ubiquitin to a lysine residue in the substrate through an isopeptide bond. The transfer of ubiquitin to the substrate is either mediated by the E3 enzyme (in the case of the HECT E3 family) or directly by E2 enzym (in the case of RING E3 family). The figure was taken from (Mabb & Ehlers 2010a).

Proteasomes and the endoplasmic reticulum-associated degradation (ERAD) pathway

Proteasomes are composed of two multiprotein complexes: the 20S core complex and the 19S regulatory complex. The 20S core complex is composed of four heptameric rings of α and β subunits and contains the proteolytic activity. The 19S complex recruits the polyubiquitylated protein, removes the ubiquitin and, using ATPase activity, unfolds the protein for translocation into the 20S chamber (Dahlmann 2005). Structurally, the 19S regulatory complex contains a hexameric ring of AAA-ATPases and ~12 non-ATPase subunits (Bochtler et al 1999, Tanahashi et al 1999).

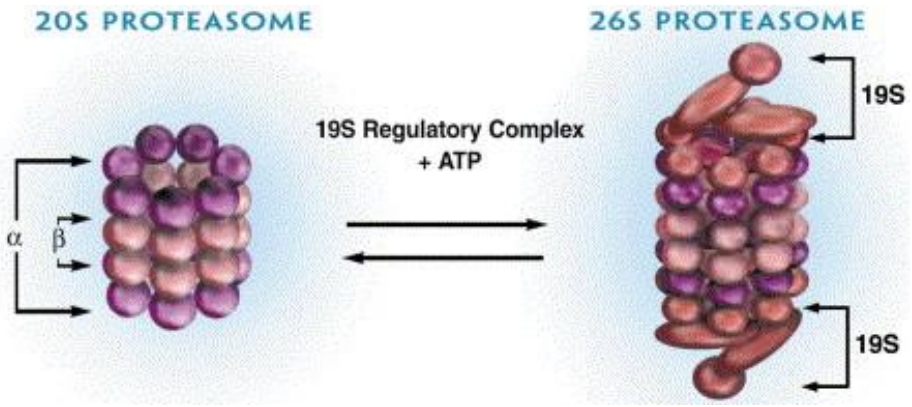

Figure 5: Proteasome structure. The proteasome is a multiprotein assembly containing the 20S core complex and the 19S regulatory complex. The 20S complex is a hollow cylinder composed of two outer α-rings and two inner β-rings, which contains the proteolytic activity. The 19S regulatory complex consists of a hexameric ring of AAA-ATPases and ~12 non-ATPase subunits (Adams 2003).

Proteasomal degradation of membrane proteins is mediated via the ERAD pathway. The most well established function of ERAD is the elimination of newly synthesized membrane proteins present in the ER, which are damaged or misfolded (Petaja-Repo et al 2001). This process is initiated by recognition of the protein to be degraded and can take place through different pathways. For example, it can be a result of a too long association with ER chaperones or of a modified glycan handling (Parodi 2000, Trombetta & Parodi 2003). Once the membrane protein is selected for ERAD, it is removed from the ER membrane into the cytoplasm by a process called retrotranslocation, which involves the action of multiple proteins (Fig. 5). First ERAD substrates are recognized by specialized UBX domain containing adaptor proteins and ubiquitinated by a membrane-associated E3 ligase (such as Hrd1). Then the ubiquitinated substrate is removed from the ER membrane in an ATP-dependent manner by the AAA-ATPase VCP/p97 and transferred to the cytoplasm for degradation by the proteasome (Lilley & Ploegh 2005, Neuber et al 2005, Ruggiano et al 2014, Schuberth & Buchberger 2005).

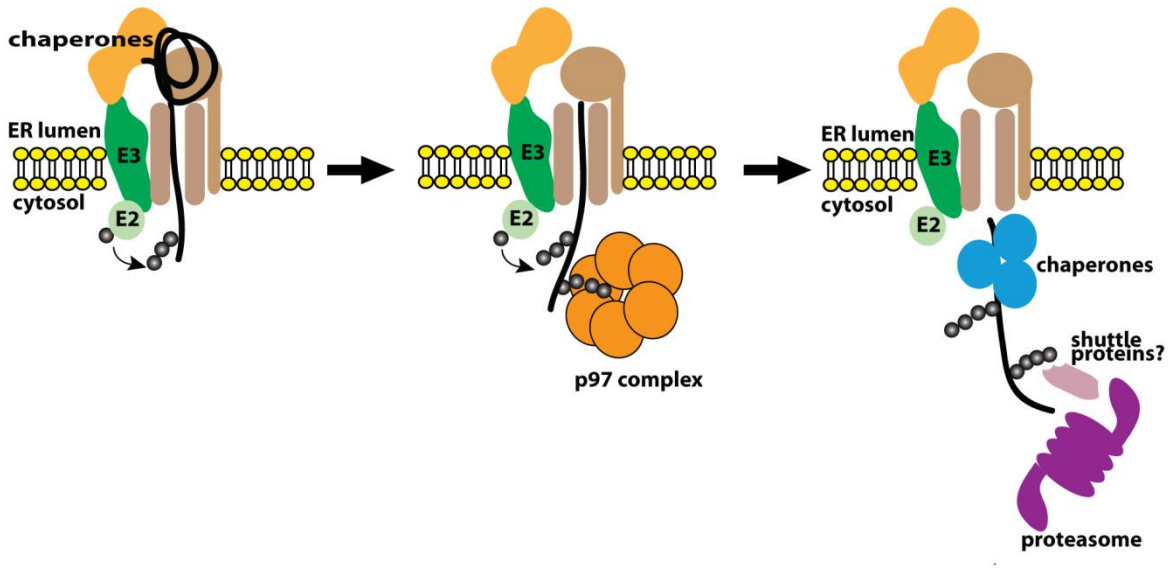

Figure 6: The ERAD pathway. First, ERAD substrates are recognized by cytoplasmic and luminal chaperones like Hsp70-family members, calnexin or calreticulin. In a second step, the protein is polyubiquitinated by a membrane bound E3 ligase (e. g. Hrd1) and removed from the ER membrane to the cytosol by a process named retrotranslocation, involving the AAA-ATPase VCP/p97. Then the polyubiquitynated protein is recognized and degraded by the proteasome. The figure was taken from (Tsai & Weissman 2011).

Lysosomes and the endosomal complex required for transport (ESCRT).

Receptors degraded via the lysosomal pathway are sorted after internalization from early endosomes to late endosomes and multivesicular endosomes (MVEs) and are then delivered to lysosomes, where they are degraded by hydrolases (Raiborg et al 2003). Sorting of an endocytosed membrane protein to lysosomes is mediated by the ESCRT machinery (Katzmann et al 2001). The ESCRT machinery consists of four multiprotein complexes ESCRT-0, -I, -II and –III. They sequentially facilitate sorting of ubiquitinated proteins from early to late endosomes and assists in the formation of intraluminal vesicles containing the cargo proteins of multivesicular endosomes (Katzmann et al 2001). Upon fusion of the MVE with the lysosome, the intraluminal vesicles are degraded by lipases and proteases present in the lysosome (Saftig & Klumperman 2009). The highly complex components of the ESCRT machinery are structurally well-established (Raiborg & Stenmark 2009). Proteins of ESCRT-0, ESCRT-I and ESCRT-II complexes contain ubiquitin interaction motifs (UIM), which allow them to interact with ubiquitin-tagged proteins (Fisher et al 2003). A specialty of the ESCRT machinery is its ability to form vesicles that bud into the endosome lumen. ESCRT-III, recruited by ESCRT-II, is thought to be mainly involved in this process of "inward budding" (Raiborg & Stenmark 2009). In addition, ESCRT-III recruits deubiquitinating enzymes (DUBs), which are important for recycling of ubiquitin (Amerik et al 2000, Swaminathan et al 1999). DUBs recruited to ESCRT-0 are assumed to reverse ubiquitination of proteins not destined for degradation (Raiborg & Stenmark 2009).

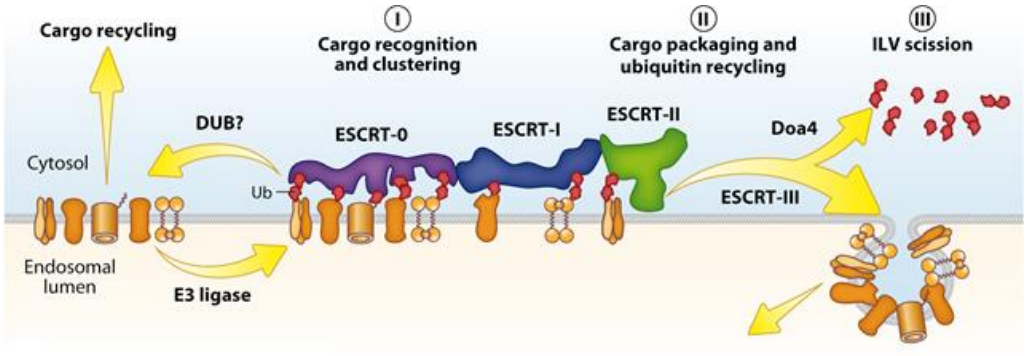

Figure 7: Marking and sorting of membrane proteins into intraluminal vesicles. Endocytosed membrane proteins are tagged with ubiquitin via an E3 Ligase. This process can be reversed via deubiquitinating enzymes (DUBs), which lead to cargo recycling. Ubiquitin-tagged proteins are recognized and clustered via the endosomal complex required for sorting (ESCRT) complexes 0, I, and II. ESCRT-III is supposed to be responsible for the packaging of cargo into intraluminal vesicles (ILVs) and their subsequent scission into the endosome. DUBs that are recruited via ESCRT-III recycle ubiquitin, which then can again be linked to proteins. The figure was taken from (MacGurn et al 2012)

Experimental Section

AIMS OF THE THESIS

The availability of $GABA_B$ receptors in the neuronal plasma membrane is determined by their rate of forward trafficking from the ER to the cell surface as well as their rates of endocytosis, recycling and degradation. Currently, there is little known about the mechansisms involved in degrading $GABA_B$ receptors and how they are regulated. Plasma membrane receptors can potentially be degraded by proteasomes or lysosomes. Differential ubiquitination is the basis for sorting the proteins into the two distinct pathways (Hershko & Ciechanover 1998, Katzmann et al 2002). While K48-linked ubiquitination is a common signal for proteasomal degradation, K63-linked ubiquitination often serves as a sorting signal for lysosomal degradation. In this thesis, we analyzed whether and under what conditions $GABA_B$ receptors are degraded by proteasomes and lysosomes. In addition, we tested the hypothesis that degradation of $GABA_B$ receptors regulates the amount cell surface receptors available for signaling. The research program is divided into three sub-projects:

Study 1: Endoplasmic reticulum-associated degradation (ERAD) controls cell surface expression of $GABA_B$ receptors

Here we tested whether $GABA_B$ receptors are degraded by proteasomes. We found that $GABA_B$ receptors in the ER are constitutively degraded by proteasomes via the ERAD machinery, which required K43-linked ubiquitination of K767/771 in the C-terminal domain of $GABA_{B2}$. The data suggest that proteasomal degradation of $GABA_B$ receptors via ERAD determines the amount of $GABA_B$ receptors present in the ER available for forward trafficking to the cell surface.

Study 2: Proteasomal degradation of $GABA_B$ receptors is mediated by the interaction of the $GABA_{B2}$ C-terminus with the proteasomal ATPase Rpt6 and regulated by neuronal activity

In this study we searched for proteins involved in proteasomal degradation of $GABA_B$ receptors and tested the hypothesis that proteasomal degradation may depend on the level of neuronal activity. We found that the C-terminus of $GABA_{B2}$ interacts with the proteasomal AAA-ATPases Rpt6. The interaction of $GABA_{B2}$ with Rpt6 and correspondingly the proteasomal degradation of the receptors were found to be regulated by neuronal activity. This mechanism adjust the amount of cell surface $GABA_B$ receptors according to the activity level of the neuron and most likely plays an important role in homeostatic plasticity.

Study 3: CaMKII-dependent K63-linked ubiquitination of $GABA_{B1}$ drives lysosomal degradation of $GABA_B$ receptors

Here we tested whether K63-linked ubiquitination of $GABA_B$ receptors serves as a sorting signal for their lysosomal degradation. We identified four lysine residues in $GABA_{B1}$ which are required to be K63-linked ubiquitinated in a CaMKII-dependent manner for lysosomal degradation of $GABA_B$ receptors. The data indicate that the activity level of CaMKII regulates K63-linked ubiquitination of $GABA_{B1}$ and thereby the rate of lysosomal degradation of $GABA_B$ receptors.

STUDY I:

Endoplasmic Reticulum Associated Degradation (ERAD) Controls Cell Surface Expression of $GABA_B$ Receptors

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ABSTRACT

Metabotropic $GABA_B$ receptors are crucial for controlling the excitability of neurons by mediating slow inhibition in the CNS. The strength of receptor signaling depends on the number of cell surface receptors, which is thought to be regulated by trafficking and degradation mechanisms. While the mechanisms of $GABA_B$ receptor trafficking are studied to some extent, it is currently unclear whether receptor degradation actively controls the number of $GABA_B$ receptors available for signaling. Here we tested the hypothesis that proteasomal degradation contributes to the regulation of $GABA_B$ receptor expression levels. Blocking proteasomal activity in cultured cortical neurons considerably enhanced total and cell surface expression of $GABA_B$ receptors, indicating the constitutive degradation of the receptors by proteasomes. Proteasomal degradation required K48-linked polyubiquitination of lysines 767/771 in the C-terminal domain of the $GABA_{B2}$ subunit. Inactivation of these ubiquitination sites increased receptor levels and $GABA_B$ receptor signaling in neurons. Proteasomal degradation was mediated by endoplasmic reticulum (ER)-associated degradation (ERAD) as shown by the accumulation of receptors in the ER upon inhibition of proteasomes, by the increase of receptor levels as well as receptor signaling upon blocking ERAD function and by the interaction of $GABA_B$ receptors with the essential ERAD components Hrd1 and p97. In conclusion, the data support a model in which the fraction of $GABA_B$ receptor available for plasma membrane trafficking is regulated by degradation via the ERAD machinery. Thus, modulation of ERAD activity by changes in physiological conditions may represent a mechanism to adjust receptor numbers and thereby signaling strength.

INTRODUCTION

Signaling strength of neurotransmitter receptors is significantly controlled by the number of receptors in the plasma membrane. Protein synthesis, cell surface trafficking, endocytotic removal from the plasma membrane and degradation of the receptors need to be precisely balanced to maintain an appropriate level of cell surface receptors. These mechanisms thus provide means for adapting receptor numbers in response to plastic changes in neurons. There is accumulating evidence that regulated protein degradation via the ubiquitin-proteasome system plays an important integrative role in synaptic plasticity (Cajigas et al 2010, Hegde , Mabb & Ehlers 2010b). Proteasomal degradation at the endoplasmic reticulum (ER) is crucial for the quality control of newly synthesized receptors. Incorrectly folded and misassembled receptor proteins are efficiently eliminated from the endoplasmic reticulum via the ER-associated degradation (ERAD) (Vembar & Brodsky 2008). Defective receptor proteins are polyubiquitinated, exported from the ER membrane and degraded by proteasomes in the cytoplasm. There is evidence that ERAD may also be involved in the regulation of the number of functional receptors in response to physiological stimuli. Prolonged activation of IP_3 receptors, which release Ca^{2+} from the ER, down-regulates the expression of the receptors in ER membranes via ERAD-dependent proteasomal degradation (Wojcikiewicz et al 2009). This is thought to be a homeostatic response to counterbalance excessive accumulation of Ca^{2+} in the cytoplasm. However, it is currently unclear whether the ERAD machinery contributes to the regulation of the cell surface density of neurotransmitter receptors.

$GABA_B$ receptors are G protein-coupled receptors assembled from the two subunits $GABA_{B1}$ and $GABA_{B2}$. They mediate slow inhibitory neurotransmission in the CNS and are thought to be involved in a variety of neurological disorders (Bettler et al 2004b). It is meanwhile well established that $GABA_B$ receptors are endocytosed from the plasma membrane via the classical dynamin and clathrin-dependent pathway and are eventually degraded in lysosomes (Benke 2010). Lysosomal targeting appears to be mediated by the ESCRT (endosomal sorting complex required for transport) machinery (Kantamneni et al 2008b) that sorts mono- and K63-linked polyubiquitinated proteins to lysosomes (Raiborg & Stenmark 2009). It is currently unclear whether proteasomal degradation contributes to the regulation of $GABA_B$ receptors available for signal transduction. Therefore, we tested in this study the hypothesis that cell surface levels of $GABA_B$ receptors might be controlled by proteasomal degradation.

EXPERIMENTAL PROCEDURES

Antibodies . The following primary antibodies were used: rabbit $GABA_{B1a,b}$ (Grampp et al 2008b, Maier et al 2010a) directed against the C terminus of $GABA_{B1}$ (affinity-purified, 1:500 for in-cell Western assay and immunofluorescence), rabbit $GABA_{B2N}$ (Grampp et al 2008b, Maier et al 2010a) directed against the N terminus of $GABA_{B2}$ (affinity-purified, 1:250 for in-cell Western assay and immunofluorescence, 1:50 for *in situ* PLA), guinea pig $GABA_{B2}$ (1:1000 for immunofluorescence in neurons and 1:4000 in HEK 293 cells, 1:1000 for Western blotting, Chemicon International), mouse PDI (1:1000 for immunofluorescence, Santa Cruz Biotechnology), mouse ubiquitin (P4D1, 1:50 for Western blotting, Santa Cruz Biotechnology), mouse ubiquitin Lys48-specific (clone Apu2, 1:50 for *in situ* PLA; Millipore), mouse VCP (p97) (1:50 for *in situ* PLA, 3E8DC11, Abcam), mouse actin (1:1000 for in-cell Western assay, Chemicon International), mouse HA (1:500 for immunofluorescence, Santa Cruz Biotechnology), rabbit SYVN1/Hrd1 (1:50 for *in situ* PLA, Bioss). Secondary antibodies were coupled either to horseradish peroxidase (1:5000, Jackson ImmunoResearch), Alexa Fluor 488 (1:1000, Invitrogen), Cy-3 (1:500, Jackson ImmunoResearch), IRDye680 (1:400, Li-COR Biosciences) or IRDye800CW (1:400, Li-COR Biosciences).

Drugs – Baclofen (50 μM, Tocris Bioscience), betulinic acid (20 μg/ml, Sigma-Aldrich), bicucullin (4 μM, Tocris Bioscience), Eeyarestatin I (5 μM, Chembridge), CNQX (2 μM, Tocris Bioscience), lactacystin (50 μM, Sigma Aldrich), MG132 (10 μM, Sigma-Aldrich), pyrenebutyric acid (50 μM, Sigma-Aldrich), SMI-UPS14 (5 μM, BostonBiochem), TTX (0.5 μM, Tocris Bioscience).

Plasmids . The following cDNAs in the appropriate expression vectors were used: $GABA_{B(1a)}$ (Kaupmann et al 1997) (pcDNA1), $GABA_{B2}$ (Kaupmann et al 1998) (pcI) (pcDNA1, $GABA_B$ plasmids were kindly provided by Dr. B. Bettler, University of Basle and Dr. K. Kaupmann, Novartis, Basle), ubiquitin and ubiquitin (K48R) (Lim et al 2005) (pRK5-HA, Addgene plasmids 17604, 17608), VCP/p97-EGFP and VCP/p97(DKO)-EGFP (Tresse et al 2010) (pEGFP-N1, Addgene plasmids 23971, 23974).

Mutation of $GABA_{B2}$. Lysines 767 and 771 in $GABA_{B2}$ were mutated to arginines using the Quick change II XL site directed mutagenesis kit from Stratagene according to the manufacturer's instructions.

Culture and transfection of cortical neurons . Primary neuronal cultures of cerebral cortex were prepared from E18 embryos of time-pregnant Wistar rats as described previously (Grampp et al 2008b, Maier et al 2010a). Neurons were kept in culture for 12 to 17 days before used. Neurons were transfected with plasmid DNA using magnetofection as detailed by Buerli et al. (Buerli et al 2007).

Culture and transfection of HEK 293 cells . HEK (Human Embryonic Kidney) 293 cells were cultured in minimum essential medium (MEM, Invitrogen) containing 10% fetal calf serum (Invitrogen), 2 mM glutamine (Q, Invitrogen) and 4% gentamicin (Invitrogen). HEK 293 cells were transfected with plasmids using the calcium phosphate precipitation method.

Proteasome activity assay . Neurons cultured in 96-well plates were incubated for 12 h with either 10 μM MG132, 50 ⌈M lactacystin or 20 μM betulinic acid followed by determination of proteasome activity using the Proteasome Glo Chymotrypsin-like cell based assay (Promega) according to the manufactures instructions.

Immunoprecipitation and Western blotting . Immunoprecipitation of $GABA_B$ receptors from deoxycholate extracts of rat brain membranes and Western blotting for the detection of $GABA_{B2}$ and ubiquitin was done as described previously (Grampp et al 2008b, Grampp et al 2007a).

Immunocytochemistry and confocal laser scanning microscopy . Double labeling immunocytochemistry was performed with cortical neurons cultured on coverslips as described previously (Grampp et al 2008b, Grampp et al 2007a, Maier et al 2010a). Neurons were analyzed by confocal laser scanning microscopy (LSM510 Meta; Zeiss, 100x plan apochromat oil differential interference contrast objective, 1.4 NA) at a resolution of 1024 x 1024 pixels in the sequential mode. Quantification of fluorescence signals and image processing was done as detailed in (Maier et al 2010a). Images shown represent a single optical layer.

In-cell Western assay . The in-cell Western assay was exactly done as in (Maier et al 2010a). Neurons cultured in 96-well plates were treated with the drug to be tested for the indicated time at 37 °C and 5% CO_2. After fixation and permeabilization, the neurons were incubated simultaneously with $GABA_B$ receptor and actin antibodies. Non-specific $GABA_B$ receptor antibody binding was determined in parallel cultures by competition using the respective peptide-antigen (10 μg/ml). After incubation with the appropriate secondary antibodies the fluorescence was measured with the Odyssey Infrared Imaging System (LI-

COR Biosciences). Specific $GABA_B$ signals were normalized to the actin signal determined in parallel.

In situ proximity ligation assay (in situ PLA) . The *in situ* PLA technology is a highly sensitive antibody-based method for the microscopic detection of protein-protein interactions and posttranslational protein modifications in cultured cells and tissue section (Leuchowius et al 2010, Soderberg et al 2006). For *in situ PLA* we used Duolink PLA probes and detection reagents according to the manufactures instructions (Olink Bioscience). The specificity of the PLA signal was validated for each pair of antibodies in HEK 293 cell expressing or not expressing $GABA_B$ receptors. In addition, in neurons omitting one of the primary antibodies did not generate PLA signals.

For signal quantification, cells were imaged for $GABA_B$ receptor expression and PLA signals by confocal microscopy (LSM510 Meta; Zeiss, 100x plan apochromat oil differential interference contrast objective, 1.4 NA, resolution 1024 x 1024 pixels, sequential mode). $GABA_B$ receptor fluorescence intensities, PLA spots and the cell area were quantified using ImageJ (http://rsbweb.nih.gov/ij/). PLA signals were normalized to the $GABA_B$ receptor signal and the cell area.

Electrophysiology . Cortical neurons at 13-15 days *in vitro* were recorded in the whole-cell voltage-clamp configuration at room temperature. Total spontaneous postsynaptic currents (sPSCs) were recorded at a holding potential of -60 mV. Baclofen-evoked potassium currents were elicited using a 10 second pulse of 50 ⌈M baclofen at -90 mV. Patch electrodes were filled with 120 mM CsCl / KCl, 10 mM EGTA, 10 mM HEPES (pH 7.4), 4 mM $MgCl_2$, 0.5 mM GTP and 2 mM ATP. Spontaneous PSCs recordings were performed using intracellular CsCl, whereas the potassium currents were recorded using an intracellular solution containing KCl. The external solution contained 140 mM NaCl, 10 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES pH 7.4 and 10 mM glucose. Potassium currents were recorded in the presence of TTX (0.5 μM), CNQX (2 μM) and bicuculline (4 μM). To enhance the amplitude of the baclofen-evoked currents, the potassium concentration of the extracellular solution was increased to 30 mM and the sodium concentration was reduced to 120 mM (to keep osmolarity constant) before the application of the $GABA_B$ agonist. All the synaptic events displaying amplitudes above the background noise (5-12 pA) were identified and analyzed off-line using MiniAnalysis 6.0.7 software (Synaptosoft). Mean amplitudes and frequency values were obtained from 1 min epoch's recordings on each experimental condition and normalized to the control condition of the individual neuron.

Statistical analysis . Data are presented as mean ± S.E.M.. The statistical analysis of data was performed with the GraphPad Prism 5 software. Unpaired t-test was used for comparing two conditions and one way ANOVA followed by Dunnett's post-hoc test for analysis of multiple conditions. The level of significance and n values are indicated in the figure legends. Differences were considered statistically significant when $p< 0.05$.

RESULTS

***The expression level of $GABA_B$ receptors is controlled by proteasomal degradation*.** It is currently unknown whether the ubiquitin-proteasome system contributes to the regulation of $GABA_B$ receptor expression levels in neurons. To gain evidence for a potential degradation of $GABA_B$ receptors by proteasomes, we treated cultured cortical neurons for 12 h with the proteasome inhibitors MG132 or lactacystin and determined the $GABA_{B1}$ and $GABA_{B2}$ protein expression levels. Under these conditions, MG132 and lactacystin decreased proteasomal activity to 31±2% and 17±1% of untreated controls, respectively (Fig. 1*A*). Both drug treatments increased total $GABA_B$ receptor expression levels (MG132, $GABA_{B1}$: 131±2 %, $GABA_{B2}$: 143±5 %; lactacystin, $GABA_{B1}$: 142±4 %, $GABA_{B2}$: 147±2 % of control; Fig. 1*B*), suggesting that under basal conditions $GABA_B$ receptors were constitutively degraded to a certain extent by proteasomes.

Prolonged inhibition of proteasomes depletes the pool of free ubiquitin (Melikova et al 2006, Patnaik et al 2000), which might also affect ubiquitin-dependent processes unrelated to proteasomal degradation. There is some evidence that $GABA_B$ receptors are sorted to lysosomes via the ubiquitin-dependent ESCRT (endosomal sorting complex required for transport) machinery (Kantamneni et al 2008b). Hence, prolonged inhibition of proteasomes might indirectly compromise lysosomal degradation of the receptors. However, an indirect contribution of lysosomal degradation could be ruled out. Pharmacologically increasing proteasome activity by treating cortical neurons for 12 h with the proteasome activator betulinic acid (Huang et al 2007), which enhanced proteasomal activity to 143±17% of control (Fig. 1*A*), significantly decreased $GABA_B$ receptor levels ($GABA_{B1}$: 69±2%, $GABA_{B2}$: 64±3% of control; Fig. 1*C*).

It has recently been shown that inhibition of the proteasome-associated deubiquitinating enzyme USP14 enhanced the degradation of proteasome substrates (Lee et al 2010). Inhibition of USP14 by incubation of cortical neurons with SMI-USP14 (small molecule inhibitor of USP14) strongly reduced $GABA_B$ receptor levels ($GABA_{B1}$: 49±4%, $GABA_{B2}$:

29±2% of control, Fig. 1*D*), further supporting the view that $GABA_B$ receptors are degraded by proteasomes.

Finally, we assessed the functional consequences of decreased $GABA_B$ receptor levels after enhancing proteasomal activity with betulinic acid by measuring spontaneous synaptic activity in electrophysiological experiments. Activation of $GABA_B$ receptors with the selective agonist baclofen considerably decreased the amplitude as well as the frequency of spontaneous postsynaptic currents (sPSCs) to 43±4% and 56±7%, respectively (Fig. 1*E*). Treatment of cultures for 12 h with betulinic acid diminished baclofen-induced inhibition of sPSCs (amplitude: from 43±4% to 90±12% of control, frequency: from 56±7% to 94±15% of control; Fig. 1*E*), supporting the hypothesis that enhanced proteasomal activity leads to reduced levels of functional $GABA_B$ receptors available for neuronal inhibition.

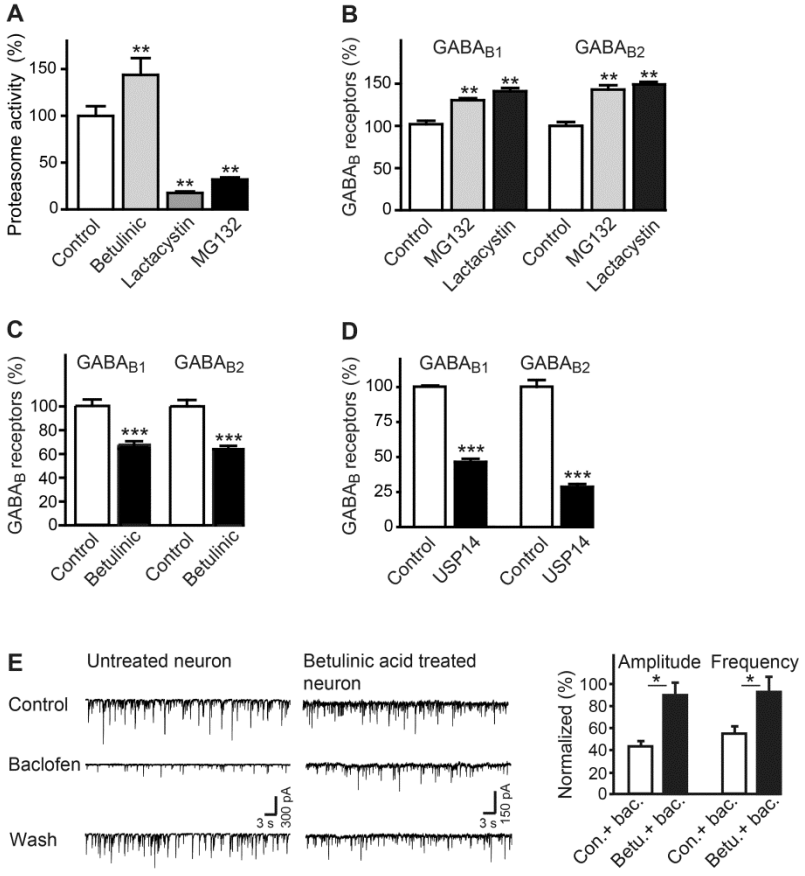

FIGURE 1. **The expression level of $GABA_B$ receptors is controlled by proteasomes.**
A, Verification of drug effects on proteasome activity. Neurons were incubated for 12 h with the indicated drugs and tested for proteasome activity. n=12 cultures; **, $p<0.01$, ANOVA.
B, Blocking proteasome activity increased the level of $GABA_B$ receptors. Neurons were incubated for 12 h with drugs and $GABA_B$ receptor levels were determined using the in-cell Western assay. Untreated neurons served as a control. n=40 cultures, **, $p<0.01$; ***, $p<0.001$, ANOVA.

C, Enhancing proteasome activity decreased the level of $GABA_B$ receptors. Neurons were incubated for 12 h with betulinic acid, followed by determination of $GABA_B$ receptor levels using the in-cell Western assay. n=40 cultures, ***, $p<0.0001$, t-test.
D, Inhibition of the deubiquitinating enzyme USP14 decreased the expression level of $GABA_B$ receptors. Neurons were incubated for 12 h with SMI-USP14 (USP14) and tested for $GABA_{B1}$ and $GABA_{B2}$ levels using the in-cell Western assay. n=20-27 cultures, ***, $p<0.0001$, t-test.
E, Enhancing proteasome activity diminished baclofen-induced inhibition of spontaneous postsynaptic currents (sPSCs). Left: Representative current traces showing sPSCs recorded from untreated cultured cortical neurons or from neurons treated for 12 h with betulinic acid. Right: Normalized amplitude and frequency values of the sPSCs. Mean amplitudes and frequency values were normalized to the control condition of the individual neuron. Con.: control, bac.: baclofen, betu.: betulinic acid. n=6, *, $p < 0.05$, t-test.

$GABA_B$ receptors undergo K48-linked polyubiquitination. K48-linked polyubiquitination of proteins serves as a signal for proteasomal degradation. Consistent with poly-ubiquitination, $GABA_B$ receptors immunoprecipitated from deoxycholate extracts of crude rat brain membranes exhibited on Western blots ubiquitin immunoreactivity in the high molecular range (Fig. 2*A*). This suggests that $GABA_B$ receptors are ubiquitinated under basal conditions to a certain extent. Likewise, using the *in situ* proximity ligation assay (PLA), we found that $GABA_B$ receptors in cultured cortical neurons display K48-linked polyubiquitination, which was considerably increased upon inhibition of proteasomal activity with MG 132 (172±11% of control, Fig. 2*B*). This indicates the accumulation of K48-linked polyubiquitinated $GABA_B$ receptors destined for proteasomal degradation.

Next we tested whether preventing K48-linked polyubiquitination affects $GABA_B$ receptor levels. Over-expression in neurons of a K48-chain elongation-defective ubiquitin mutant, in which lysine (K) 48 had been exchanged for an arginine (R) (Ub(K48R)), considerably increased the level of $GABA_B$ receptors ($GABA_{B1}$: 166±7%, $GABA_{B2}$: 140±7% of control, Fig. 2*C*). This finding corroborates a K48-linked polyubiquitin-mediated proteasomal degradation of $GABA_B$ receptors.

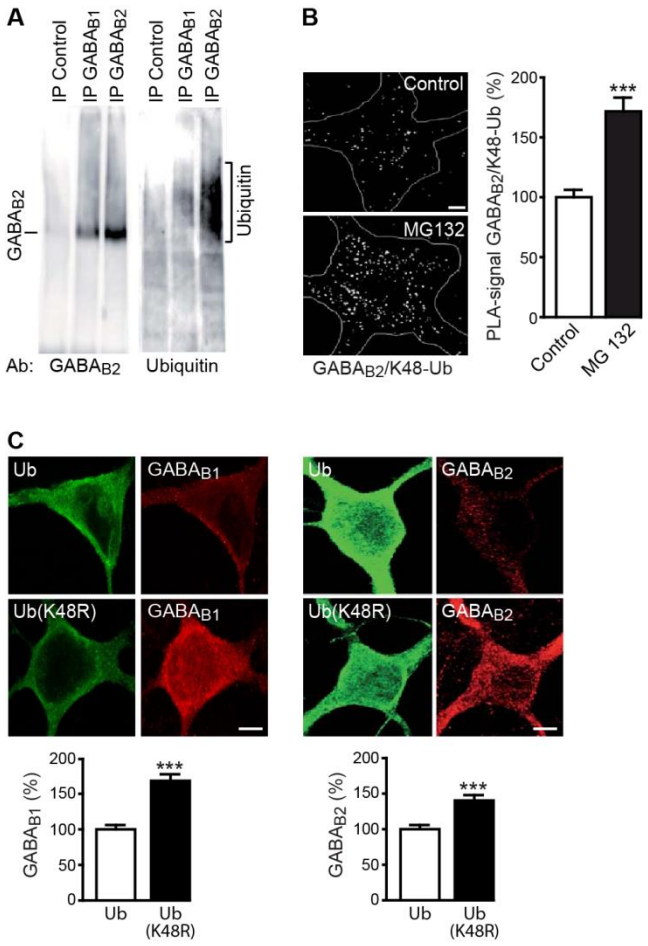

Figure 2. **K48-linked ubiquitination controls the expression level of $GABA_B$ receptors.**
A, Demonstration of ubiquitination of native $GABA_B$ receptors. $GABA_B$ receptors were immunoprecipitated from deoxycholate extracts of rat brain membranes using either $GABA_{B1a,b}$ or $GABA_{B2N}$ antibodies. The immunoprecipitate was subjected to Western blotting for detection of $GABA_{B2}$ and ubiquitin. The high molecular smear detected with the ubiquitin antibody is typical for polyubiquitinated proteins. The ubiquitin signal in the $GABA_{B1}$ immunoprecipitate was considerably weaker than in the $GABA_{B2}$ immunoprecipitate because the $GABA_{B1a,b}$ antibody beads were less efficient in precipitating $GABA_B$ receptors than the $GABA_{B2}$ antibody beads. Specificity of the immunoprecipitation was verified with non-immune antibodies (control). IP, immunoprecipitate; Ab, antibody.

The C-terminal domain of $GABA_{B2}$ contains a major K48-linked polyubiquitination site . An *in silico* analysis predicted two lysines in the C-terminal domain of $GABA_{B2}$ at position 767 and 771 as likely candidates for ubiquitination. We inactivated these potential ubiquitination sites by exchanging both lysines for arginines ($GABA_{B2}$(RR)) (Fig. 3*A*). Upon transfection into HEK 293 cells, $GABA_{B2}$(RR) displayed reduced K48-linked polyubiquitination (61±6% of wild type; Fig. 3*B*), indicating that K767/771 is a main site for K48-linked polyubiquitination in $GABA_{B2}$.

We then tested whether $GABA_{B1}$ is also a target for K48-linked polyubiquitination. However, HEK 293 cells transfected with $GABA_{B1}$ showed only marginal $GABA_{B1}$/K48-

linked ubiquitination PLA signals as compared to HEK 293 cells expressing $GABA_{B1}$ and $GABA_{B2}$ (12±6%, Fig. 3*C*). In line with this finding, coexpression of $GABA_{B1}$ with $GABA_{B2}$(RR) yielded a similar reduction in $GABA_B$ receptor/K48-linked polyubiquitination signals (56±8%, Fig. 3*C*) as observed for $GABA_{B2}$(RR) alone (61±6%, Fig. 3*B*). Thus, $GABA_{B2}$ appears to be the main target for K48-linked polyubiquitination of $GABA_B$ receptors.

Over-expressing $GABA_{B2}$(RR) in cultured neurons increased $GABA_B$ receptor levels to a similar level as observed after chronic proteasome inhibition ($GABA_{B1}$: 152±15%, $GABA_{B2}$: 146±9% of control; Fig. 3*D*). This suggests that K767/771 in $GABA_{B2}$ is the major K48-linked polyubiquitination site required for proteasomal degradation of $GABA_B$ receptors.

The functional consequence of the increased $GABA_{B2}$ cell surface density after transfecting neurons with $GABA_{B2}$(RR) was analyzed by measuring baclofen-induced K^+ currents using whole-cell patch-clamp recordings. Transfection of $GABA_{B2}$(RR) in neurons resulted in 2.8±0.6-fold increased K^+ channel current amplitudes after activation of $GABA_B$ receptors with baclofen as compared to neurons transfected with wild type $GABA_{B2}$ (Fig. 3*E*). Thus, preventing proteasomal degradation of $GABA_{B2}$ by over-expression of $GABA_{B2}$(RR) increased the number of functional cell surface $GABA_B$ receptors available for signaling.

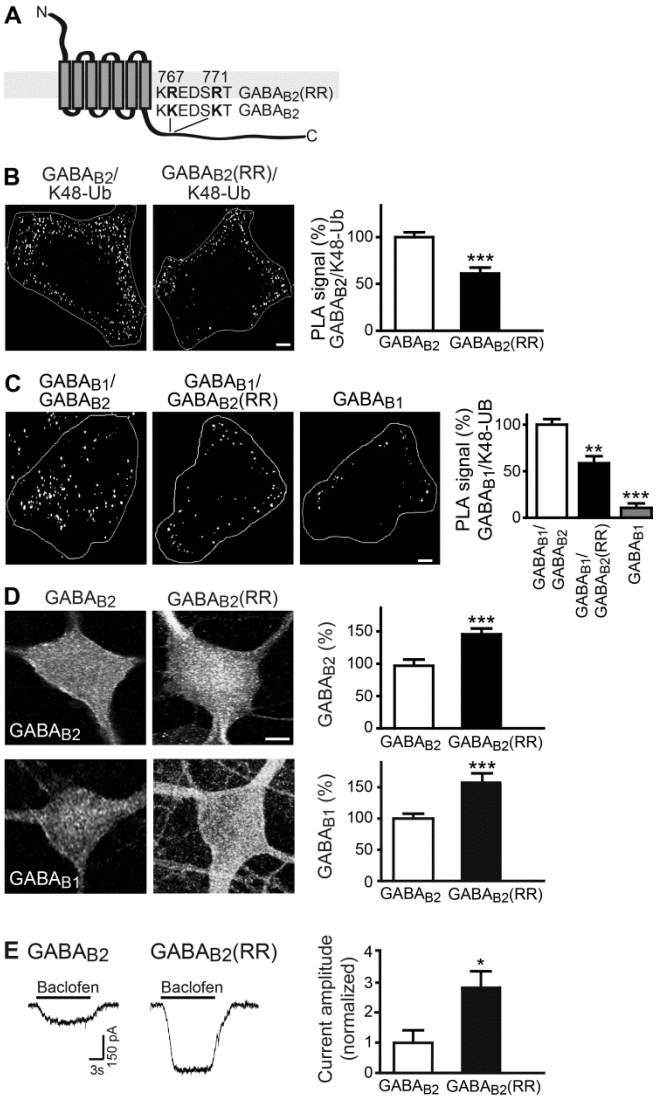

FIGURE 3. **The C-terminal domain of $GABA_{B2}$ contains a major K48-linked ubiquitination site.**
A, Scheme depicting the location of mutated lysines in $GABA_{B2}$.
B, Decreased K48-linked polyubiquitination of a $GABA_{B2}$ mutant in which lysines 767 and 771 had been changed to arginines ($GABA_{B2}$(RR)). HEK293 cells were transfected with plasmids containing either $GABA_{B2}$ or $GABA_{B2}$(RR) together with HA-Ub plasmid. Cells were analyzed by *in situ* PLA using antibodies directed against $GABA_{B2}$ and K48-linked polyubiquitin to detect K48-linked polyubiquitinated $GABA_{B2}$ (left, white dots, scale bar: 5 µm). Right: quantification of PLA signals. n=30 cells, *******, p<0.0001, t-test.
C, $GABA_{B2}$ is the main target for K48-linked polyubiquitination. HEK 293 cells were transfected with plasmids containing cDNA for ubiquitin and either $GABA_{B1}$ alone, $GABA_{B1}$ and wild type $GABA_{B2}$ or $GABA_{B1}$ and $GABA_{B2}$(RR). Cells were analyzed by *in situ* PLA to detect K48-linked polyubiquitinated $GABA_B$ receptors (left, white dots, scale bar: 5 µm). Right: quantification of PLA signals. n=25-30 cells, ******, p<0.001, *******, p<0.0001, ANOVA.
D, Increased $GABA_B$ receptor expression levels in neurons over-expressing $GABA_{B2}$(RR). Neurons were co-transfected with plasmids containing GFP and $GABA_{B2}$ or GFP and $GABA_{B2}$(RR) and stained for $GABA_{B2}$ (left, upper panels) or $GABA_{B1}$ (left, lower panels). Right: quantification of fluorescence signals. n=27 ($GABA_{B1}$) and 40 ($GABA_{B2}$) neurons, ***, p=0.0003; t-test. Scale bar: 10 µm.
E, Over-expression of $GABA_{B2}$(RR) in neurons increased $GABA_B$ receptor-mediated K^+ currents. Left: Representative traces of baclofen-induced K^+ currents recorded in neurons transfected with wild type $GABA_{B2}$ or $GABA_{B2}$(RR). Right: Normalized K^+ current amplitudes. Current amplitude of $GABA_{B2}$(RR) transfected neurons were normalized to the mean of current amplitudes recorded from

$GABA_{B2}$ transfected neurons. bac.: baclofen. n=16 for $GABA_{B2}$ and n=18 for $GABA_{B2}$(RR), *, $p < 0.05$, t-test.

Cell surface expression of $GABA_B$ receptors is regulated by ERAD. The most likely mechanism for proteasomal degradation of $GABA_B$ receptors is the ER-associated degradation (ERAD). If $GABA_B$ receptors are degraded by ERAD, inhibition of proteasomal activity should result in an accumulation of $GABA_B$ receptors in the ER. Indeed, blocking proteasomal activity in neurons for 12 h with MG132 increased the number of $GABA_{B2}$ clusters (136±6% of control) as well as the clusters co-localizing with a marker protein for the ER (protein disulfide isomerase [PDI], 133±7% of control, Fig. 4*A*).

To further establish the role of ERAD in regulating cellular $GABA_B$ receptor levels we tested the effect of directly inhibiting ERAD. Treatment of neurons for 12 h with the ERAD inhibitor Eeyarestatin I (EerI) (Fiebiger et al 2004, Wang et al 2008) increased both total $GABA_{B2}$ (183±15% of control, Fig. 4*B*) and cell surface levels of $GABA_{B2}$ (204±32% of control, Fig. 4*C*). Over-expression of $GABA_{B2}$(RR), which lack the main K48-linked polyubiquitination sites, did not further increase total (112±7% of control, Fig. 4*D*) or cell surface $GABA_B$ receptor levels (93±15% of control, Fig. 4*E*). These observations indicate that K48-linked polyubiquitinated $GABA_B$ receptors are degraded by ERAD.

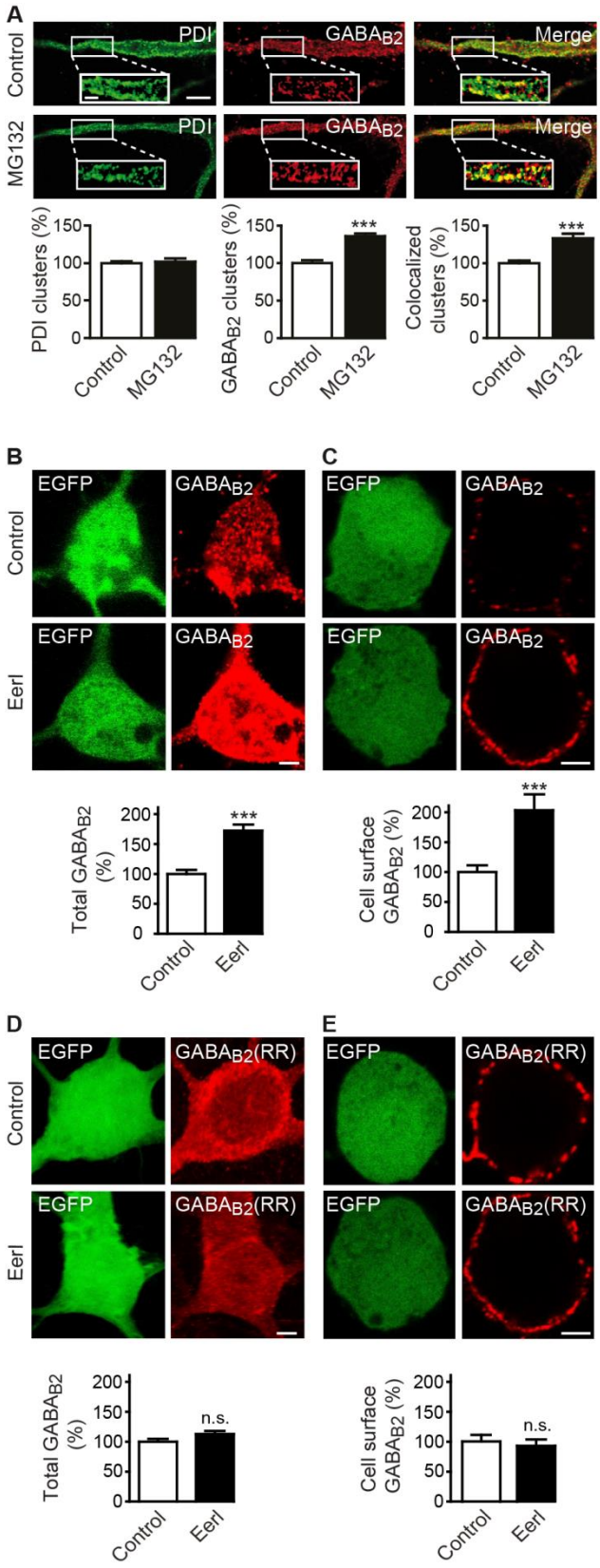

FIGURE 4. **$GABA_B$ receptors are degraded via the ERAD pathway.**
A, Blocking proteasomal activity increased the number of $GABA_{B2}$ clusters co-localized with the ER marker protein PDI. Neurons were incubated for 12 h with MG132 and stained for $GABA_{B2}$ (red) and PDI (green). The yellow clusters in the merged image indicate the co-localization of $GABA_{B2}$ and PDI (scale bars: 5 μm, 1 μm for insets). Lower panels: quantification revealed enhanced co-localization of

$GABA_{B2}$ and PDI after proteasome inhibition. Control refers to the number of clusters in neurons not treated with MG132. n=25-30 neurons, ***, p<0.0001; t-test.
B-E, Blocking the ERAD pathway increased the level of $GABA_B$ receptors. Neurons were transfected with plasmids containing EGFP (for detection of transfected neurons) and either wild type $GABA_{B2}$ (*B, C*) or $GABA_{B2}$(RR) (*D, E*). After 48 h cultures were incubated for 12 h with or without (controls) the ERAD blocker Eeyarestatin I (EerI). Total (*B, D*) and cell surface (*C, E*) $GABA_B$ receptor levels were determined immunocytochemically using $GABA_{B2}$ antibodies (red, upper panels). Scale bars: 10 µm. Lower panels: quantification of $GABA_{B2}$ fluorescence signals. n=28-30 neurons; ***, p<0.0001, n.s., p>0.05; t-test.

$GABA_B$ receptors interact with the ERAD E3 ubiquitin ligase Hrd1. Hrd1 is one prototypical ERAD E3 ubiquitin ligases responsible for K48-linked polyubiquitination of ERAD substrates (Smith et al 2011). Using *in situ* PLA, we further confirmed the potential degradation of $GABA_B$ receptors via ERAD by showing that $GABA_B$ receptors interact with Hrd1 (Fig. 5*A*). Inhibition of ERAD for 12 h with EerI increased the number of interactions ($GABA_{B2}$/Hrd1: 490±45%, $GABA_{B1}$/Hrd1: 305±18% of control; Fig. 5*A*), indicating the accumulation of $GABA_B$ receptors at this central ERAD multiprotein complex. In line with this observation, blocking ERAD function for 12 h with EerI considerably increased the level of K48-linked polyubiquitinated $GABA_B$ receptors (242±21% of control, Fig. 5*B*).

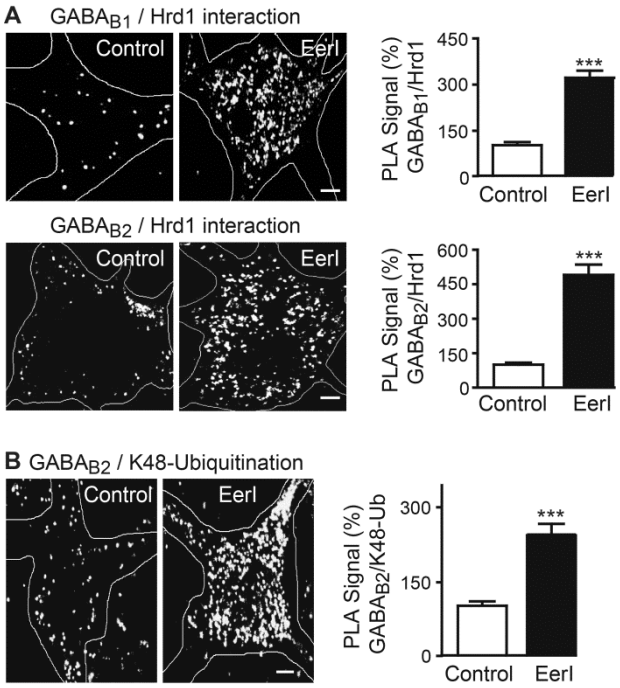

FIGURE 5. **$GABA_B$ receptors interact with the ERAD E3 ligase Hrd1.**
A, Demonstration of the interaction of $GABA_B$ receptors with the ERAD E3 ligase Hrd1 using *in situ* PLA with Hrd1 and $GABA_{B2}$ antibodies (left, upper panels) or $GABA_{B1}$ antibodies in cortical neurons (left, lower panels). Treatment of neurons for 12 h with EerI strongly increased the number of

interactions. Right: quantification of *in situ* PLA signals. n=21-27 neurons, ***, p<0.0001; t-test. Scale bar: 5 μm.
B, Inhibition of ERAD induced the accumulation of K48-linked polyubiquitinated $GABA_B$ receptors. Neurons were incubated with EerI for 12 h and analyzed for K48-linked ubiquitination using *in situ* PLA (white dots in representative images, scale bar: 5 μm). Right: quantification of *in situ* PLA signals. n=32 cells, ***, *p*<0.0001, t-test.

$GABA_B$ receptors interact with the essential ERAD component p97. The AAA-ATPase p97 is a central constituent of the ERAD machinery involved in the retrotranslocation of proteins to the cytoplasm for proteasomal degradation (Wang et al 2004). Using *in situ* PLA, we found that $GABA_B$ receptors interact with p97 (Fig. 6*A*). This finding further demonstrates the ERAD-mediated degradation of $GABA_B$ receptors. Inhibition of p97 by EerI decreased the interaction of $GABA_{B2}$ with p97 (40±8% of control; Fig. 6*A*), suggesting that the association is activity-dependent.

Inhibition of p97 function in neurons by over-expression of a dominant-negative mutant of p97 (p97[DKO]) considerably increased total (176±11% of control, Fig. 6*B*) as well as cell surface $GABA_B$ receptor levels (143±11% of control; Fig. 6*C*) as compared to neurons over-expressing wild type p97. Over-expressing in addition $GABA_{B2}$(RR) did not further increase total (wild type p97: 100±6%, p97(DKO): 104±6%, Fig. 6*D*) or cell surface $GABA_B$ receptor levels (wild type p97: 100±12%, p97(DKO): 100±11%, Fig. 6*E*), indicating that ubiquitination of $GABA_{B2}$ is required for being recognized by the ERAD machinery.

Whole-cell patch-clamp recordings finally verified that inhibition of ERAD function by over-expression of p97(DKO) increased the level of functional cell surface $GABA_B$ receptors (Fig. 6*F*). Neurons over-expressing p97(DKO) displayed considerably increased amplitudes of baclofen-induced K^+ currents (control: 72±14 pA, p97(DKO): 139±14 pA; Fig. 7 *F*). These experiments show that $GABA_B$ receptors are degraded by ERAD, which affects the levels of total and cell surface $GABA_B$ receptors.

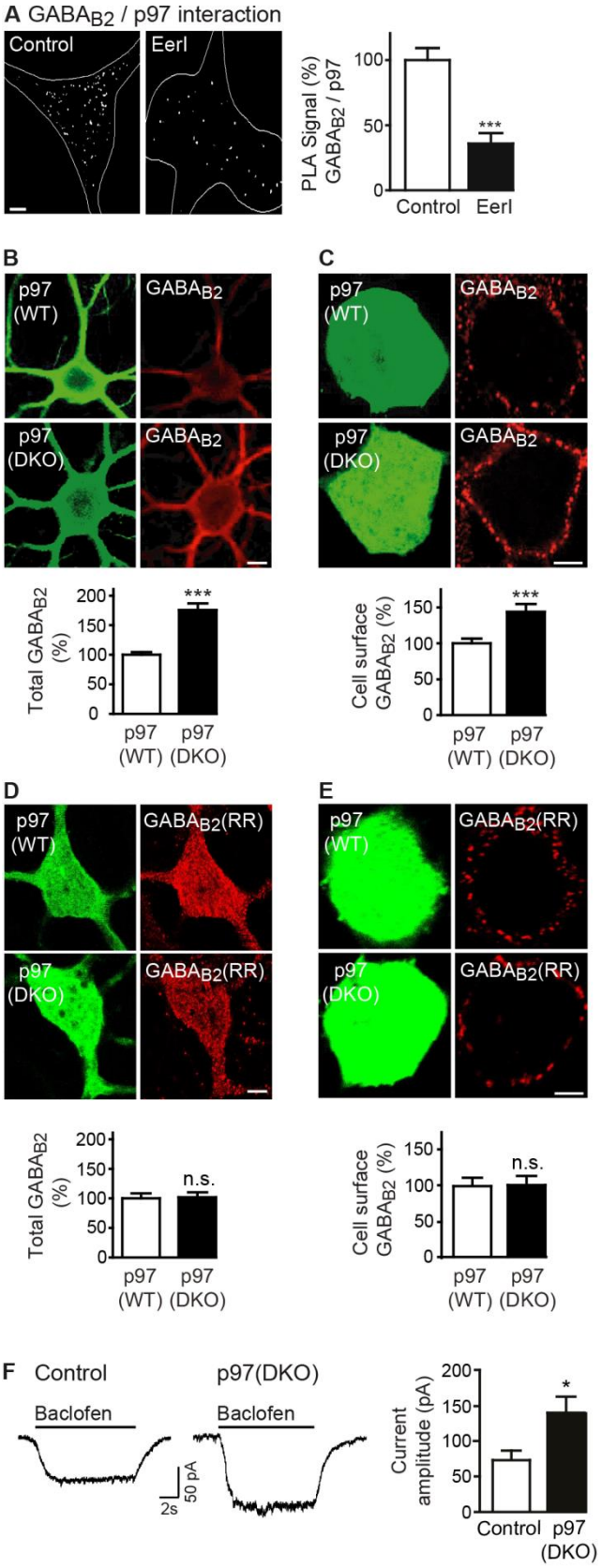

FIGURE 6. **GABA$_B$ receptors interact with the ERAD AAA-ATPase p97.**
A, Demonstration of the interaction of GABA$_B$ receptors with the ERAD AAA-ATPase p97 in cortical neurons using *in situ* PLA. Treatment of neurons for 12 h with the p97 inhibitor EerI strongly reduced

the interaction. Left: quantification of *in situ* PLA signals. n=18-21 neurons, ***, p<0.0001; t-test. Scale bar: 5 μm.

B-C, Disruption of ERAD function by over-expression of a dominant-negative mutant of p97 enhanced the level of total (*B*) and cell surface $GABA_{B2}$ receptors (*C*). Neurons were transfected with plasmids containing HA-tagged p97 or its dominant-negative mutant HA-tagged p97(DKO) and stained for $GABA_{B2}$ (red) and HA (green). Scale bars: 10 μm. Lower panels: quantification of $GABA_{B2}$ fluorescence signals. n=40-50 neurons, ***, p<0.0001; t-test.

D-E, Over-expression of $GABA_{B2}$(RR) in neurons transfected with wild type p97 or p97(DKO) did not result in an additional increase of total (*D*) or cell surface (*E*) $GABA_{B2}$ levels. Neurons were transfected with plasmids containing $GABA_{B2}$(RR) and either HA-tagged p97 or its dominant-negative mutant HA-tagged p97(DKO) and stained for $GABA_{B2}$ (red) and HA (green). Scale bars: 10 μm. Lower panels: quantification of $GABA_{B2}$ fluorescence signals. n=28-30 neurons; n.s., p>0.05; t-test.

F, Disruption of ERAD by over-expression of p97(DKO) in neurons increased $GABA_B$ receptor-mediated K^+ currents. Neurons were transfected either with plasmids containing EGFP (control) or with plasmids containing p97(DKO). Left: Representative traces of baclofen-induced K^+ currents. Right: K^+ current amplitudes. n=10 for control and n=8 for p97(DKO), *, $p < 0.05$, t-test.

DISCUSSION

Mechanisms controlling the cell surface density of $GABA_B$ receptors are of pivotal importance for determining the level of $GABA_B$ receptor-mediated neuronal inhibition. Because $GABA_B$ receptors control glutamatergic neurotransmission (Chalifoux & Carter 2011a) modulation of their cell surface density is presumed to significantly contribute to synaptic plasticity. However, the mechanisms that control cell surface expression of $GABA_B$ receptors are largely unknown. In the present study, we identified proteasomal degradation via the ER-resident ERAD machinery as a mechanism that determines cell surface expression of $GABA_B$ receptors.

Our data indicate that a fraction of $GABA_B$ receptors in the ER is constitutively K48-linked polyubiqutinated and degraded by the ERAD machinery. This conclusion is based on the observation that blocking proteasomal activity, inhibiting ERAD function or interfering with $GABA_B$ receptor K48-linked polyubiquitination increased the expression levels of $GABA_B$ receptors in neurons. Lysines 767/771 in the C-terminal domain of $GABA_{B2}$ appear to represent the main K48-linked polyubiquitination sites required for proteasomal degradation as their mutational inactivation rendered $GABA_B$ receptors largely immune to degradation. It is currently unclear whether K48-linked polyubiquitination of both lysines or only of K767 or K771 serves as a tag for proteasomal degradation. A recent proteomic study analyzing the ubiquitination state of rat brain synaptic proteins identified K771 in $GABA_{B2}$ as being ubiquitinated (Na et al 2012). This observation favors K771 as the main K48-linked polyubiquitination site in $GABA_{B2}$.

There are several lines of evidence that in particular $GABA_B$ receptors residing in the ER are degraded by proteasomes via ERAD. First, upon blocking proteasomal activity the receptors accumulated in the ER. Second, blocking ERAD function pharmacologically or by over-expressing a dominant-negative mutant of the AAA-ATPase p97, which mediates the retrotranslocation of proteins to the cytoplasm for proteasomal degradation (Wang et al 2004), increased $GABA_B$ receptor levels. Third, $GABA_B$ receptors interacted with the ERAD proteins p97 and Hrd1. Hrd1 is the prototypical ERAD E3 ligase (Smith et al 2011) and most likely one of the ubiquitin ligases that mediate ubiquitination of $GABA_B$ receptors because stalling proteasomal degradation considerably increased its interaction with $GABA_B$ receptors and the level of K48-linked polyubiquitinated $GABA_B$ receptors.

In all cases tested, $GABA_{B1}$ and $GABA_{B2}$ were concomitantly up- or down-regulated to a similar extent, suggesting that assembled $GABA_B$ receptor complexes are degraded by ERAD. This notion is further strengthened by the finding that 1) inactivation of the

ubiquitination sites in $GABA_{B2}$ increased the expression levels of $GABA_{B1}$ and $GABA_{B2}$ as well as $GABA_B$ receptor-activated K^+ current amplitudes, 2) that interfering with ERAD function increased $GABA_B$ receptor function (baclofen-induced K^+ currents) and 3) that both $GABA_{B1}$ and $GABA_{B2}$ generated *in situ* PLA signals with the ERAD E3 ubiquitin ligase Hrd1 although only K48-linked polyubiquitination of K767/771 in $GABA_{B2}$ appears to be required for proteasomal degradation of the receptors.

What might be the physiological implications of this mechanism? The most firmly established function of ERAD is the degradation aberrant proteins in the ER (Brodsky 2012). In addition, ERAD has been shown to rapidly degrade activated IP_3 receptors in the ER to prevent excessive elevation of cytosolic Ca^{2+} concentrations (Wojcikiewicz et al 2009), indicating that ERAD may also contribute to the regulation of functional receptors. Because blocking ERAD increased the level of functional $GABA_B$ receptors and ERAD appears to degrade assembled heterodimeric receptors it is rather unlikely that the role of ERAD is simply the degradation of un- or misfolded $GABA_B$ receptor subunits. The constitutive degradation of $GABA_B$ receptors suggests that ERAD controls the amount of receptors available for cell surface trafficking. This view is supported by recent studies on the regulation of cell surface $GABA_A$ receptors. Chronic suppression of neuronal activity or inhibition of L-type voltage-gated calcium channels decreased the level of functional $GABA_A$ receptors in the neuronal plasma membrane by a mechanism dependent on the ubiquitination of the $GABA_A$ receptor □□-subunit and proteasome activity, most likely via the ERAD pathway (Saliba et al 2009, Saliba et al 2007). These findings imply that regulation of ERAD activity is a potential mechanism to adjust the level of functional $GABA_B$ receptors to changing physiological condition. Our finding that modulation of proteasomal activity up- or down-regulates the level of functional $GABA_B$ receptors supports this view. Interestingly, the level of proteasomal activity correlates with the activity state of neurons (Djakovic et al 2009). We therefore hypothesize that the amount of functional $GABA_B$ receptors inserted into the plasma membrane is regulated by neuronal activity via ERAD.

STUDY II:

Proteasomal Degradation of gamma-Aminobutyric Acid B Receptors is Mediated by the Interaction of the $GABA_{B2}$ C Terminus with the Proteasomal ATPase Rpt6 and Regulated by Neuronal Activity

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ABSTRACT

Regulation of cell surface expression of neurotransmitter receptors is crucial for determining synaptic strength and plasticity but the underlying mechanisms are not well understood. We previously showed that proteasomal degradation of $GABA_B$ receptors via the endoplasmic reticulum (ER) associated protein degradation (ERAD) machinery determines the number of cell surface $GABA_B$ receptors and thereby $GABA_B$ receptor-mediated neuronal inhibition. Here we show that proteasomal degradation of $GABA_B$ receptors requires the interaction of the $GABA_{B2}$ C terminus with the proteasomal AAA-ATPase Rpt6. A mutant of Rpt6 lacking ATPase activity prevented degradation of $GABA_B$ receptors but not the removal of Lys48-linked ubiquitin from $GABA_{B2}$. Blocking ERAD activity diminished the interaction of Rpt6 with $GABA_B$ receptors resulting in increased total as well as cell surface expression of $GABA_B$ receptors. Modulating neuronal activity affected proteasomal activity and correspondingly the interaction level of Rpt6 with $GABA_{B2}$. This resulted in altered cell surface expression of the receptors. Thus, neuronal activity-dependent proteasomal degradation of $GABA_B$ receptors by the ERAD machinery is a potent mechanism regulating the number of $GABA_B$ receptors available for signaling and is expected to contribute to homeostatic neuronal plasticity.

INTRODUCTION

A prominent characteristic of neuronal plasticity is the regulation of the number of neurotransmitter receptors available for signaling (Turrigiano 2008). The operative process can include regulation of protein synthesis, cell surface trafficking, endocytotic removal from the plasma membrane or degradation of the receptors. It is now well recognized that protein degradation via the ubiquitin-proteasome system plays a key role in synaptic plasticity (Cajigas et al 2010, Hegde 2010, Mabb & Ehlers 2010b). For instance, pharmacological modulation of neuronal activity in cultured hippocampal neurons induced a remodeling of postsynaptic proteins, which was dependent on proteasome-mediated protein degradation (Ehlers 2003). On the receptor level, chronic elevation of neuronal activity has been shown to downregulate AMPA receptors (Fu et al 2011) and NMDA receptors (Ehlers 2003, Kato et al 2005) in a proteasome-dependent manner as a homeostatic response.

The most well established role for proteasomal degradation of membrane receptors is the quality control of newly synthesized receptors in the endoplasmic reticulum (ER). Folding and assembly of receptors is a rather inefficient process frequently resulting in incorrectly folded and misassembled proteins. Defective receptor proteins are Lys^{48}-linked polyubiquitinated, exported from the ER membrane and degraded by proteasomes. This process is executed by a multiprotein machinery called ER associated protein degradation (ERAD) (Vembar & Brodsky 2008). In addition to its quality control function, ERAD may also be involved in the activity-dependent regulation of neurotransmitter receptors. For instance, the level of functional $GABA_A$ receptors in the plasma membrane has been shown to be downregulated after suppression of neuronal activity or blocking L-type voltage-gated Ca^{2+} channels by a mechanism that involved ubiquitination of the $GABA_A$ receptor β3 subunit and proteasomes (Saliba et al 2009, Saliba et al 2007). Pulse-chase experiments in combination with inhibiting ER-Golgi transport indicated that newly synthetized $GABA_A$ receptors in the ER were degraded most likely by ERAD.

The excitability of neurons is controlled, amongst others, by the G protein coupled $GABA_B$ receptors. $GABA_B$ receptors are heterodimers composed of the two subunits $GABA_{B1}$ and $GABA_{B2}$. They mediate slow inhibitory neurotransmission by activating K^+ channels and inhibiting Ca^{2+} channels (Bettler et al 2004b). We recently showed that a fraction of $GABA_B$ receptors is Lys^{48}-linked ubiquitinated at $Lys^{767/771}$ located in the C-terminal domain of the $GABA_{B2}$ subunit and constitutively degraded by ERAD (Zemoura et al 2013a). This mechanism controls the pool of assembled $GABA_B$ receptors in the ER destined for forward trafficking to the plasma membrane and consequently determines the level of $GABA_B$

receptor-mediated inhibition. In this study we addressed the unresolved questions which proteasomal component interacts with $GABA_B$ receptors and whether ERAD-mediated degradation of $GABA_B$ receptors is regulated by changes in neuronal activity.

EXPERIMENTAL PROCEDURES

Antibodies : Rabbit $GABA_{B1a,b}$ (Grampp et al 2008b, Maier et al 2010a) directed against the C terminus of $GABA_{B1}$ (affinity-purified, 1.8 μg/ml for immunofluorescence), rabbit $GABA_{B2N}$ (Grampp et al 2008b, Maier et al 2010a) directed against the N terminus of $GABA_{B2}$ (affinity-purified, 2.6 μg/ml for whole cell ELISA and total as well as cell surface immunofluorescence staining, 13 μg/ml for *in situ* PLA), guinea pig $GABA_{B2}$ (1:1000 for Western blotting, 1:250 for *in situ* PLA, Chemicon International), mouse Rpt6 (clone p45-110, 1:1000 for immunofluorescence using HEK293 cells and 1:50 using neurons, 1:50 for Western blotting, 1:20 for *in situ* PLA, Enzo), rabbit ubiquitin Lys^{48}-specific (clone Apu2, 1:50 for *in situ* PLA; Millipore), rabbit ubiquitin Lys^{63}-specific (clone Apu3, 1:50 for *in situ* PLA; Millipore), mouse actin (1:1000 for whole cell ELISA, Chemicon International), mouse HA (1:500 for immunofluorescence, 1:200 for *in situ* PLA, Santa Cruz Biotechnology). Secondary antibodies were labeled with either horseradish peroxidase (1:5000, Jackson ImmunoResearch), Alexa Fluor 488 (1:1000, Invitrogen), Cy-3 (1:500, Jackson ImmunoResearch), IRDye680 (1:400, Li-COR Biosciences) or IRDye800CW (1:400, Li-COR Biosciences).

Drugs: CNQX (6-cyano-7-nitroquinoxaline-2,3-dione, 2 μM, Tocris Bioscience), D-AP5 (50 μM, Tocris Bioscience), Eeyarestatin I (5 μM, Chembridge), MG132 (10 μM, Sigma-Aldrich), picrotoxin (PTX, 20 μM, Tocris Bioscience), pyrenebutyric acid (50 μM, Sigma-Aldrich).

Plasmids : rat $GABA_{B(1a)}$ (Kaupmann et al 1997), rat $GABA_{B2}$ (Kaupmann et al 1998), rat $GABA_{B2T749}$ (Pagano et al 2001b) ($GABA_B$ plasmids were provided by Dr. B. Bettler, University of Basle and Dr. K. Kaupmann, Novartis, Basle), rat $GABA_{B2}$(RR) (Zemoura et al 2013a), human HA-Rpt6 and human HA-$Rpt6^{(Lys196Met)}$ (Kumar et al 2010) (gift from Dr. G. Swarup, CSIR, India), mouse p45/Rpt6 (gift from Dr. Pierre Chambon, IGBMC, University of Strasbourg), pGBT9PheS (gift from Dr. Gerald Radziwill and Dr. Karin Moelling, University of Zurich).

Yeast Two-Hybrid Assay : The sequence encoding the last 12 C terminal amino acids of rat $GABA_{B2}$ was introduced into the pGBT9PheS vector (Schneider et al 1997) and used

for screening a human brain cDNA library (Clontech) with the *Yeast* two-hybrid system using standard techniques.

Culture and Transfection of HEK 293 Cells : HEK (Human Embryonic Kidney) 293 cells were cultured in Dulbecco's Modified Eagles Medium (DMEM, Gibco, Life Technologies) containing 10% fetal bovine serum (PAA) and penicillin/streptomycin (PAA). Plasmids were introduced into HEK 293 cells using the polyethylenimine method according to the jetPEI protocol (Polyplus Transfection).

Introduction of Peptides into HEK 293 Cells : Small synthetic peptides were introduced into HEK 293 cells as described in (Takeuchi et al 2006). A synthetic peptide comprising the last 14 C terminal amino acids of $GABA_{B2}$ with 7 additional arginines for rendering it cell-permeable was generated (RRRRRRR-RHVPPSFRVMVSGL, GenScript). A peptide containing the same amino acids but in a random sequence was used as a control (RRRRRRR-RLGPHVRMFVSSVP, GenScript). Both peptides were biotinylated at their N terminus to permit detection via DyLight649-conjugated steptavidin (Jackson ImmunoResearch). Twenty-four hours after transfection with $GABA_B$ receptor and Rpt6 plasmids, the HEK 293 cells were washed with PBS and incubated for 5 min with 50 μM pyrenebutyric acid in PBS. Then the peptide was added (final concentration 10 μM), incubated for 15 min followed by washing the cells two times with PBS. After the addition of fresh culture medium, the cells were incubated for additional 24 h at 37°C / 5% CO_2 and used for immunofluorescence experiments.

Culture and Transfection of Cortical Neurons: Primary neuronal cultures of cerebral cortex were prepared from E18 embryos of Wistar rats as detailed previously (Grampp et al 2008b, Maier et al 2010a). Neurons were used after 12 to 17 days in culture. Neurons were transfected with plasmid DNA using Lipofectamine 2000 (Invitrogen) and CombiMag (OZ Biosciences) exactly as described in (Buerli et al 2007).

Proteasome Activity Assay : Neurons cultured in 96-well plates were incubated for 12 h with either 20 μM picrotoxin or 10 μM CNQX / 20 μM D-AP5 followed by determination of proteasome activity using the Proteasome Glo Chymotrypsin-like cell based assay (Promega) according to the manufactures instructions.

Whole cell ELISA *:* The whole cell ELISA was exactly done as previously (Maier et al 2010a, Zemoura et al 2013a). Neurons cultured in 96-well plates were treated with the indicated drugs for 12 hours at 37 °C and 5% CO_2. For determining total expression of $GABA_B$ receptors the neurons were fixed, permeabilized and incubated simultaneously with antibodies directed against $GABA_{B2}$ and actin. The fluorescence signals were quantified using

the Odyssey imaging system. $GABA_{B2}$ signals were normalized to the actin signal determined in parallel.

For cell surface staining, living neurons were incubated with $GABA_{B2N}$ antibodies for 2 h at 4 °C. For normalization, the cell-permeable nuclear marker DRAQ5 (1:2000, Biostatus Ltd.) was used.

Immunoprecipitation and Western Blotting : $GABA_B$ receptors were immunoprecipitated from 0.5 % deoxycholate extracts of rat brain membranes followed by Western blotting for the detection of $GABA_{B2}$ and Rpt6 as previously (Grampp et al 2008b).

Immunocytochemisty and Confocal Laser Scanning Microscopy : Double labeling immunocytochemistry on HEK 293 cells and cortical neurons was done excactly as described previously (Grampp et al 2008b, Maier et al 2010a). Images of cells were taken by confocal laser scanning microscopy (LSM510 Meta, Zeiss, 100x plan apochromat oil differential interference contrast objectitve, 1.4 NA or LSM700, Zeiss, 40x plan apochromat oil differential interference contrast objectitve, 1.4 NA) at a resolution of 1024 x 1024 pixels in the sequential mode. Quantification of fluorescence signals and image processing was done as described in (Maier et al 2010a).

In Situ Proximity Ligation Assay (in situ PLA) : The *in situ* PLA technology enables the microscopic detection of protein-protein interactions and posttranslational modifications of proteins in cells *in situ* (Leuchowius et al 2010, Soderberg et al 2006). The target proteins are detected using two primary antibodies raised in different species and a corresponding pair of oligonucleotide labeled species-specific secondary antibodies (PLA probes). Only when the two primary antibodies bound to their target proteins are located in very close proximity (< 30 nm), specific oligonucleotides can hybridize to the PLA probes enabling a rolling-cycle-amplification reaction that generates a long DNA strand to which specific fluorophore-labeled oligonucleotides are hybridized. The signal from each pair of PLA probes generates an individual fluorescent spot detectable by fluorescence microscopy.

In this study *in situ* PLA was employed for detecting the interaction of $GABA_B$ receptors with Rpt6 (using mouse Rpt6 1:20 and rabbit $GABA_{B2N}$ 13 μg/ml μg/ml), HA-tagged Rpt6 with $GABA_B$ receptors (using mouse HA 1:200 and rabbit $GABA_{B2N}$ 13 μg/ml), as well as for detecting Lys^{48}-linked (rabbit ubiquitin Lys^{48}-specific 1:50 and guinea pig $GABA_{B2}$ 1:250) and Lys^{63}-linked (rabbit ubiquitin Lys^{63}-specific 1:50 and guinea pig $GABA_{B2}$ 1:250) ubiquitination of $GABA_B$ receptors. The specificity of the PLA signal was validated for each pair of antibodies in HEK 293 cell expressing or not expressing $GABA_B$ receptors. In addition, in neurons omitting one of the primary antibodies did not generate PLA signals.

In situ PLA was performed using the Duolink In Situ kit (Olink Bioscience) exactly according to the manufacturer's instructions. Briefly, cortical neurons grown on cover slips were washed with PBS, fixed with 4% paraformaldehyde for 20 min at room temperature, washed again with PBS and permeabilised for 10 min with 0.2% Triton X-100 in PBS. After washing again with PBS, the neurons were incubated with the appropriate pair of primary antibodies (diluted in PBS containing 3% BSA) over night at 4°C. Thereafter, the cells were washed four times for 5 min with PBS, followed by incubation with the PLA probes (PLA probe anti-mouse minus and PLA probe anti-rabbit plus or PLA probe anti-guinea pig plus, all diluted 1:5 in 3% BSA/PBS) for 1 hour at 37°C. After washing the cells two times for 5 min with PBS the ligation solution diluted 1:5 in water was added to the neurons and incubated in a pre-heated humidity chamber for 1 hour at 37°C. The neurons were then washed two times for 5 min with 10 mM Tris, pH 7.4, 0.15 M NaCl, 0.05% Tween 20. Finally, the amplification solution containing the fluorescently labelled oligonucleotides diluted 1:5 in water along with secondary antibodies for the determination of the $GABA_{B2}$ expression levels was added to the neurons and incubated in a pre-heated humidity chamber for 100 min at 37°C. Subsequently, the neurons were washed two times with 0.2 M Tris, pH 7.4, 0.1 M NaCl and once with 0.002 M Tris, 0.001 M NaCl for 1 min in the dark at room temperature and mounted on microscope slides with Dako Fluorescent Mounting Medium. Stained neurons were immediately analyzed by laser scanning confocal microscopy (LSM 510 Meta, Zeiss, 100x plan apochromat oil differential interference contrast objectitve, 1.4 NA). Five optical sections were taken with a distance of 0.3 µm and a resolution of 1024x1024 pixels.

Quantification was done by counting the PLA spots within the soma of the neurons using the MacBiophotonics ImageJ software (version 1.41n). First, the area of the soma and the integrated fluorescence intensity of the $GABA_{B2}$ signal were determined and then the PLA spots were counted. PLA signals were normalized to the $GABA_{B2}$ signal and the area of the soma.

RESULTS

$GABA_{B2}$ Interacts with the Proteasomal AAA-ATPase Rpt6: We recently showed that the expression of total and cell surface $GABA_B$ receptors is regulated by proteasomes via the endoplasmic reticulum associated degradation (ERAD) (Zemoura et al 2013a). To identify proteins that might be involved in proteasomal degradation of $GABA_B$ receptors we screened a brain cDNA library with a sequence comprising the last 12 C-terminal amino acids of $GABA_{B2}$ for interacting proteins using the yeast two-hybrid assay. One of the eight putative $GABA_B$ receptor-interacting proteins detected with this system, the AAA-ATPase Rpt6/Sug1/p45 (hereafter named Rpt6), was related to protein degradation. Rpt6 is a component of the 19S regulatory particle of the proteasome and has been implicated in recruiting proteins to proteasomes for degradation (Gianni et al 2002, Inoue et al 2006, Masuyama & MacDonald 1998, Rezvani et al 2012, Su et al 2000, Wang et al 2007). We verified the interaction of Rpt6 with native $GABA_B$ receptors by their co-immunoprecipitation from rat brain extracts (Fig. 1*A*) and by *in situ* PLA in cultured neurons (Fig. 1*B*). Moreover, inhibition of proteasomal activity for 30 min with MG132 considerably increased the interaction of Rpt6 with $GABA_{B2}$ (156±19% of control, Fig. 1*B*), suggesting that the receptors are no longer degraded and remained bound to Rpt6. This finding was further corroborated by colocalization studies. Blocking proteasomal activity with MG132 for 30 min resulted in a small increase of $GABA_{B2}$ clusters (120±8% of control, Fig. 1*C*), whereas Rpt6 clusters remained unchanged. However, the co-localization of $GABA_{B2}$ clusters with Rpt6 clusters was considerably increased (193±12% of control, Fig. 1*C*). These results suggest that interaction of Rpt6 with $GABA_{B2}$ mediates proteasomal degradation of $GABA_B$ receptors.

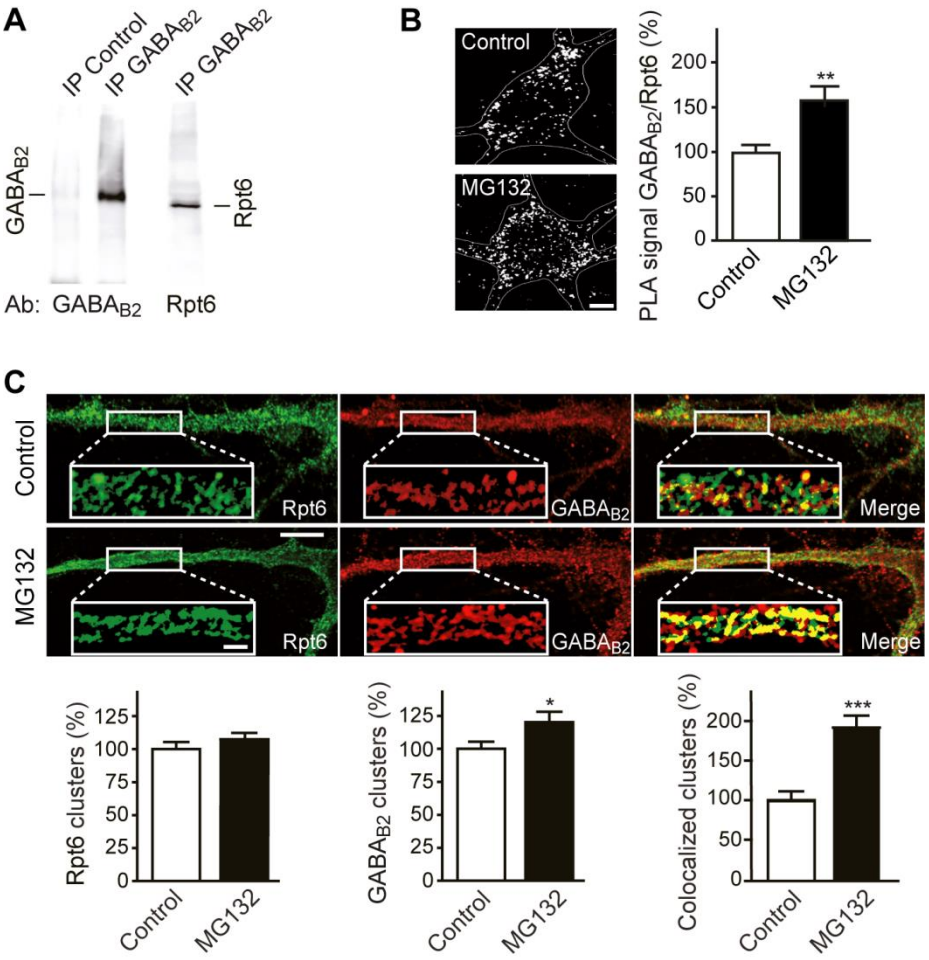

FIGURE 1. **GABA$_B$ receptors interact with the proteasomal AAA-ATPase Rpt6.**
A, coimmunoprecipitation of GABA$_B$ receptors with Rpt6. GABA$_B$ receptors were immunoprecipitated from 0.5% deoxycholate extracts prepared from rat brain membranes using GABA$_{B2N}$ antibodies coupled to protein A-agarose. The extensively washed immunoprecipitate was subjected to Western blotting for detection of GABA$_{B2}$ and Rpt6. Specificity of the immunoprecipitation was verified by using protein A-beads conjugated to non-immune antibodies (control). IP, immunoprecipitate; Ab, antibody.
B, enhanced GABA$_{B2}$-Rpt6 interaction after proteasome inhibition detected by *in situ* PLA (left, white dots). Neurons were incubated for 30 min with the proteasome inhibitor MG132 (10 µM), followed by *in situ* PLA using antibodies directed against GABA$_{B2}$ and Rpt6. The images depict the PLA signals in the soma and proximal dendrites (outlined in white). Right: quantification of *in situ* PLA signals in the soma. Means ± S.E.M., 30-36 neurons from three experiments. Scale bar: 5 µm; **, $p<0.001$, t-test.
C, increased colocalization of GABA$_{B2}$ and Rpt6 after blocking proteasomal activity in proximal dendrites. Neurons were incubated for 30 min with the proteasome inhibitor MG132 (10 µM) and stained with antibodies directed against GABA$_{B2}$ (red) and Rpt6 (green). Yellow clusters in the merged image indicate the colocalization of GABA$_{B2}$ and Rpt6 (top, scale bars: 5 µm, 1 µm for insets). Bottom: Quantification of GABA$_{B2}$ and Rpt6 colocalization after proteasome inhibition. Means ± S.E.M., 20-24 neurons from two experiments, *, $p<0.05$; ***, $p<0.0001$; t-test.

The effect of Rpt6 on $GABA_B$ receptors was analyzed in detail using coexpression experiments in HEK 293 cells. HEK 293 cells overexpressing Rpt6 displayed reduced levels of total ($GABA_{B1}$: 56±3%, $GABA_{B2}$: 49±3% of control; Fig. 2*A*) as well as cell surface $GABA_B$ receptors ($GABA_{B1}$: 38±5%, $GABA_{B2}$: 47±5% of control; Fig. 2*B*), indicating that Rpt6 mediates degradation of $GABA_B$ receptors. Co-expression of Rpt6 with individual $GABA_B$ receptor subunits reduced the expression level of $GABA_{B2}$ (60±4% of control, Fig. 2*C*) but did not affect the level of $GABA_{B1}$ (Fig. 2*D*), demonstrating that Rpt6 specifically interacts with $GABA_{B2}$ to downregulate $GABA_B$ receptors. This notion was further substantiated by the finding that the expression level of a C-terminal truncated version of $GABA_{B2}$ ($GABA_{B2}$(T749)), which does not contain the Rpt6 interaction site, was not affected by coexpression with Rpt6 (Fig. 2*E*).

Recently we showed that proteasomal degradation of $GABA_B$ receptors requires Lys^{48}-linked ubiquitination of the $GABA_{B2}$ C-terminal domain at $Lys^{767/771}$ (Zemoura et al 2013a). To analyze whether Rpt6-mediated down-regulation depends on ubiquitination of $GABA_{B2}$ we expressed Rpt6 together with a mutant of $GABA_{B2}$ in which $Lys^{767/771}$ were exchanged for arginines ($GABA_{B2}$(RR)) to prevent ubiquitination at these sites (Zemoura et al 2013a). As expected, $GABA_{B2}$(RR) was resistant to Rpt6-mediated down-regulation, verifying that Rpt6 is involved in proteasomal degradation of Lys^{48}-linked ubiquitinated $GABA_B$ receptors (Fig. 2*F*).

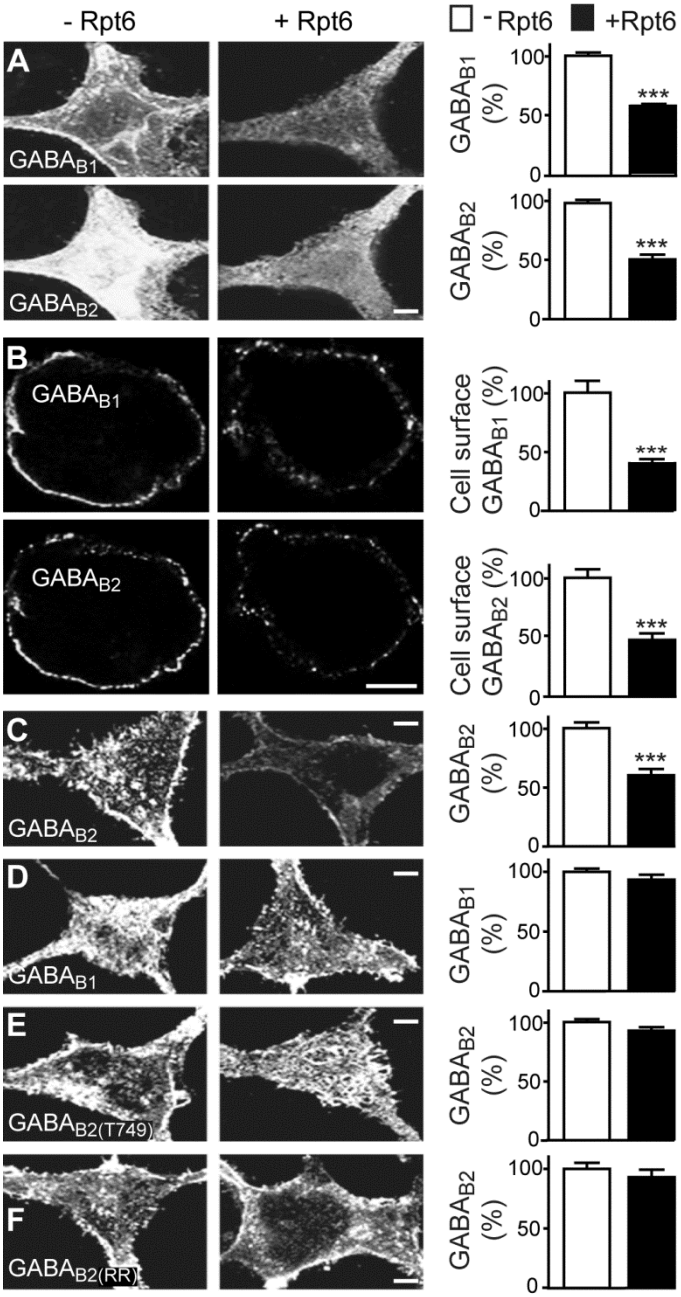

FIGURE 2. **Rpt6 mediates proteasomal degradation via interaction with the C terminus of GABA$_{B2}$.**

HEK 293 cells were transfected with plasmids containing GABA$_{B1a}$ and GABA$_{B2}$ (*A*, *B*), GABA$_{B2}$ or GABA$_{B1a}$ alone (*C*, *D*), a truncated version of GABA$_{B2}$ (GABA$_{B2}$T749) lacking the intracellular located C-terminal domain (*E*), or GABA$_{B2}$(RR), a GABA$_{B2}$ mutant with inactivated Lys^{48}-linked ubiquitination sites required for proteasomal degradation (*F*) without (left panels, -Rpt6) or with Rpt6 plasmid (right panels, +Rpt6). Forty-eight hours after transfection, the cells were stained for the indicated GABA$_B$ receptor subunit (left, representative images) and Rpt6 (not shown). Right panels: quantification of GABA$_B$ receptor staining levels. Means ± S.E.M., 23-120 cells from 2-4 experiments. ***, $p<0.0001$; t-test. Scale bar: 10 μm.

Finally, to prove that Rpt6-mediated downregulation of $GABA_B$ receptors in fact depends on interaction of $GABA_{B2}$ with Rpt6, we used a synthetic peptide (R2C-Pep) comprising the last 14 C-terminal amino acids of $GABA_{B2}$ to disrupt the $GABA_{B2}$/Rpt6 interaction. R2C-Pep inhibited the downregulation of $GABA_{B2}$ by Rpt6 (108±6% of control, Fig. 3), whereas a control peptide (R2r-Pep, random order of the same amino acids) had no significant effect (80±4% of control, Fig. 3).

These findings indicate that proteasomal degradation of $GABA_B$ receptors depends on ubiquitination of $GABA_{B2}$ and is mediated by the interaction of the $GABA_{B2}$ C terminus with Rpt6.

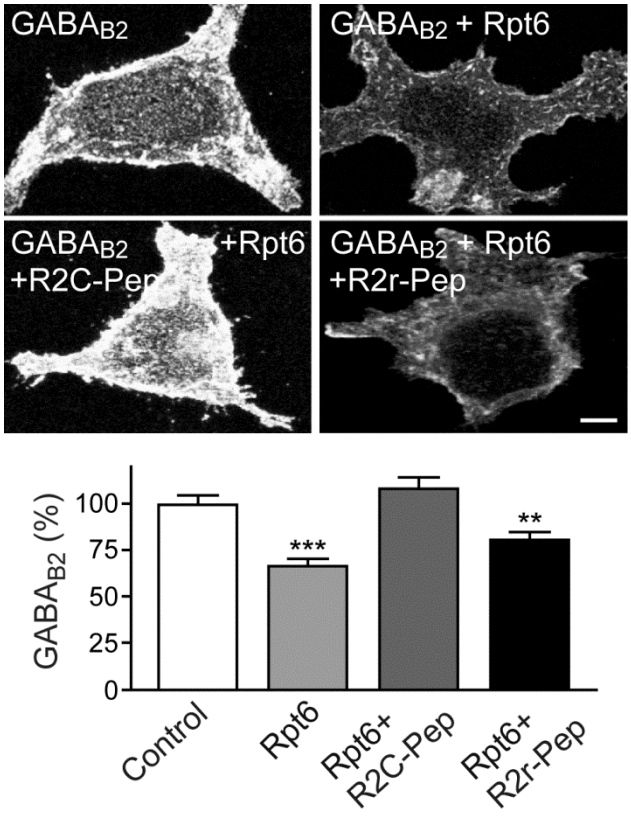

FIGURE 3. **A peptide comprising the last 14 C-terminal amino acids of $GABA_{B2}$ blocked the Rpt6-mediated downregulation of $GABA_{B2}$.**
Twenty-four hours after transfection of HEK 293 cells with plasmids containing $GABA_{B2}$ or $GABA_{B2}$ and Rpt6 (+Rpt6) a peptide comprising the last 14 C-terminal amino acids of $GABA_{B2}$ (R2C-Pep) or a peptide containing the same amino acids but in a random sequence (R2r-Pep) was added. After additional 24 h, the cells were stained for $GABA_{B2}$ (left), Rpt6 (not shown) and the peptide (not shown). Left: Representative images (scale bar: 10 μm). Right: quantification of total $GABA_{B2}$ staining. $GABA_{B2}$ staining in HEK 293 cells only expressing $GABA_{B2}$ ($GABA_{B2}$) was taken as control. Means ± S.E.M., 90-110 cells from three experiments. **, $p<0.01$; ***, $p<0.001$; one way ANOVA, Dunnett's post-hoc test.

Intact ATPase Activity of Rpt6 is Required for Proteasomal Degradation of $GABA_B$ Receptors: All six proteasomal AAA-ATPases, including Rpt6, are involved in substrate recognition, unfolding and translocation of proteins into the barrel shaped destruction chamber of the 20S proteasome (Sledz et al 2013). To test whether ATPase activity of Rpt6 is required for degradation of $GABA_B$ receptors in their native environment, we transfected neurons with either EGFP (control), Rpt6 or a mutant of Rpt6 (Rpt6(DN)) which lacks ATPase activity (mutation of Lys196 to Met) (Kumar et al 2010) and tested for total and cell surface expression of $GABA_B$ receptors (Fig. 4). Unlike overexpression of Rpt6 in HEK 293 cells, transfection of neurons with Rpt6 did not reduce total (Fig. 4*A*) or cell surface (Fig. 4*B*) expression of $GABA_B$ receptors. This might be due to a lower level of overexpression in neurons, a saturation of proteasomes with Rpt6 in neurons or to the different cellular environment. However, transfection of neurons with Rpt6(DN) significantly increased both total (150±7% of control, Fig. 4*A*) and cell surface (157±8% of control, Fig. 4*A*) $GABA_B$ receptors. This finding indicates that ATPase activity of Rpt6 is required for constitutive proteaseomal degradation of $GABA_B$ receptors.

To test whether the loss of proteasomal degradation of $GABA_B$ receptors in neurons expressing Rpt6(DN) was based on the impaired ATPase activity or on a potential inability of $GABA_{B2}$ to interact with the mutant Rpt6, we transfected neurons with either Rpt6 (control) or Rpt6(DN) and tested for interaction with $GABA_{B2}$ using *in situ* PLA. The number of interactions were similar (statistically not different) in neurons expressing wild type Rpt6 or Rpt6(DN) (Fig. 4*C*), demonstrating that the reduced proteasomal degradation for $GABA_B$ receptors in neurons expressing Rpt6(DN) was caused by the impaired ATPase activity of Rpt6(DN).

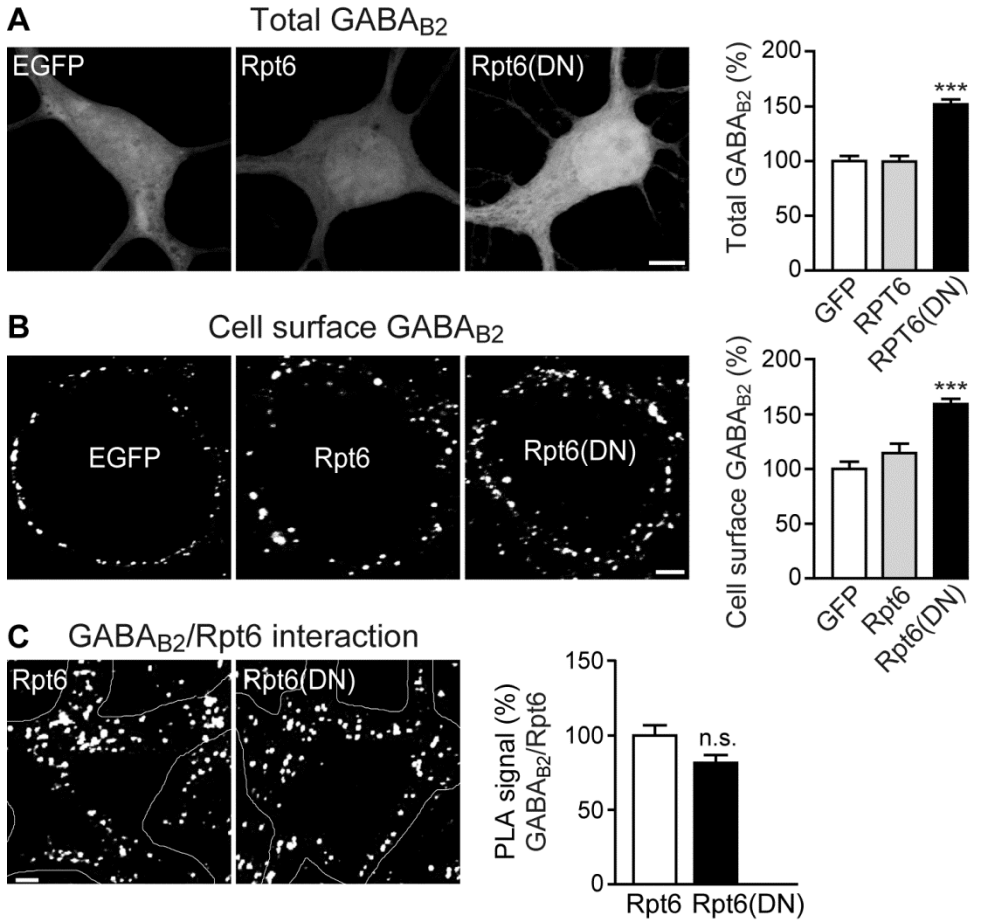

FIGURE 4. **Intact ATPase activity of Rpt6 is required for degradation of $GABA_B$ receptors.**
A-B, inactivation of ATPase activity of Rpt6 increased $GABA_B$ receptor expression levels. Neurons were transfected with either wild type Rpt6 or a non-functional mutant of Rpt6 (Rpt6(DN)) lacking ATPase activity. After 48 h, neurons were tested for total (A) and cell surface (B) expression of $GABA_{B2}$ by immunofluorescence staining. Left: Representative images (scale bar: 5 μm). Right: quantification of immunofluorescence signals. Means ± S.E.M., 30 neurons from 3 experiments. ***, $p<0.0001$; one way ANOVA, Dunnetts post-hoc test.
C, the $GABA_{B2}$/Rpt6 interaction is independent of Rpt6 ATPase activity. Neurons transfected with either wild type Rpt6 or Rpt6(DN) were tested for interaction of $GABA_{B2}$ with Rpt6 by *in situ* PLA. Left: Representative images depicting PLA signals (white dots, scale bar: 5 μm). Right: quantification of *in situ* PLA signals. Means ± S.E.M., 23 neurons from three preparations. ns, $p>0.05$; t-test.

Proteins destined for proteasomal degradation are usually tagged with Lys^{48}-linked polyubiquitin. After binding to the proteasome the protein is deubiquitinated by Rpn11 present in the 19S regulatory particle, unfolded by the proteasomal AAA-ATPases located at the base of the 19S regulatory particle and thread into the 20S proteasome for degradation (Sorokin et al 2009). Because Rpn11 activity is unlikely to be affected in neurons transfected with Rpt6(DN) we expected $GABA_B$ receptors bound to Rpt6(DN) to be deubiquitinated although they cannot be translocated into the degradation chamber of the 20S proteasome.

Using *in situ* PLA, we indeed detected a strongly reduced level of Lys^{48}-linked ubiquitinated $GABA_B$ receptors in neurons expressing Rpt6(DN) (39±3% of control, Fig. 5*A*). In contrast, Lys^{63}-linked ubiquitination of $GABA_B$ receptors, which is not involved in proteasomal degradation, was not affected in Rpt6(DN) expressing neurons (Fig. 5*B*).

These experiments indicate that $GABA_B$ receptors bound to Rpt6 are deubiquinated but cannot be degraded if the ATPase activity of Rpt6 is impaired.

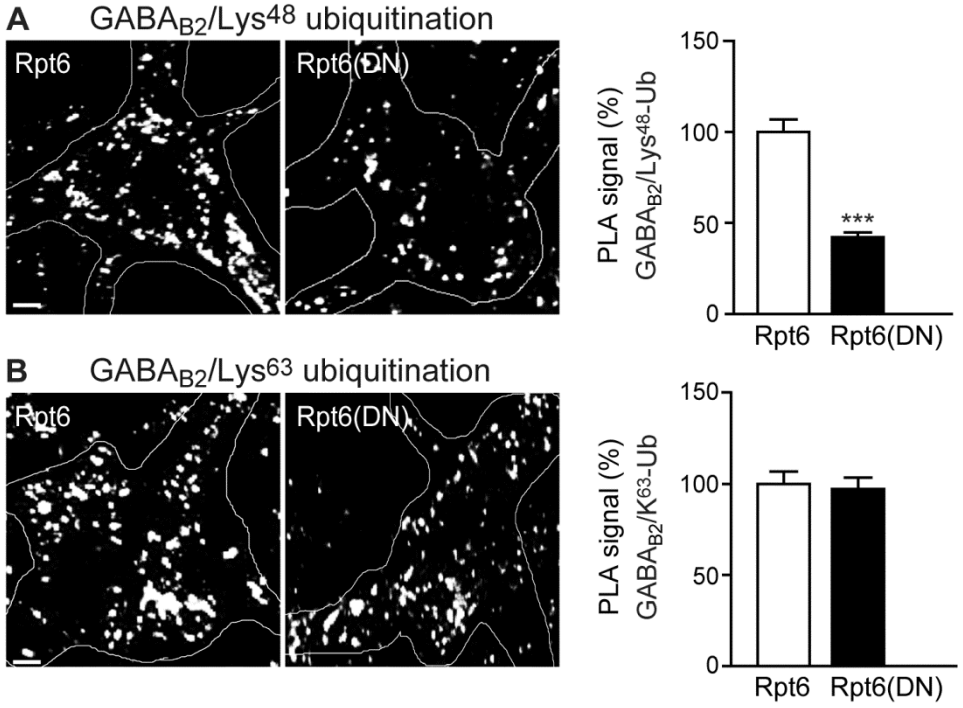

FIGURE 5. **Inactivation of Rpt6 reduced the fraction of Lys^{48}-linked ubiquitinated $GABA_B$ receptors.**
Neurons were transfected with either wild type Rpt6 or a non-functional mutant of Rpt6 (Rpt6(DN)) lacking ATPase activity. After 48 h, neurons were fixed, permeabilized and tested for Lys^{48} (*A*) and Lys^{63}-linked ubiquitination (*B*) of $GABA_B$ receptors using *in situ* PLA. Left: Representative images depicting the PLA signals (white dots, Scale bar: 5 μm). Right: quantification of *in situ* PLA signals. Means ± S.E.M., 30 (*A*) and 24 (*B*) neurons from three preparations. ***, $p<0.0001$; t-test.

Interaction of $GABA_{B2}$ with Rpt6 is Reduced upon Blocking ERAD*:* We previously showed that $GABA_B$ receptors are constitutively degraded by proteasomes via the ERAD machinery (Zemoura et al 2013a). Blocking ERAD activity using the p97 inhibitor Eeyarestatin I (EerI), which inhibits translocation of proteins from the ER membrane to the cytoplasm for proteasomal degradation (Fiebiger et al 2004, Wang et al 2008), increased both total (152±6% of control, Fig. 6*A*) as well as cell surface $GABA_B$ receptors (129±6% of control, Fig. 6*B*),

confirming our previous findings. Because Eeyarestatin I targets p97, preventing ER-exit of ubiquitinated proteins and their interaction with the proteasomes located in the cytoplasm, we expected a diminished interaction of $GABA_B$ receptors with Rpt6. We employed *in situ* PLA to test whether the increase in $GABA_B$ receptors upon blocking p97 is associated with a decreased interaction of $GABA_{B2}$ with Rpt6. Consistent with the essential role of Rpt6 for proteasomal degradation of $GABA_B$ receptors inhibition of p97 with Eeyarestatin I significantly reduced the $GABA_{B2}$/Rpt6 interaction (71±4% of control, Fig. 6*C*).

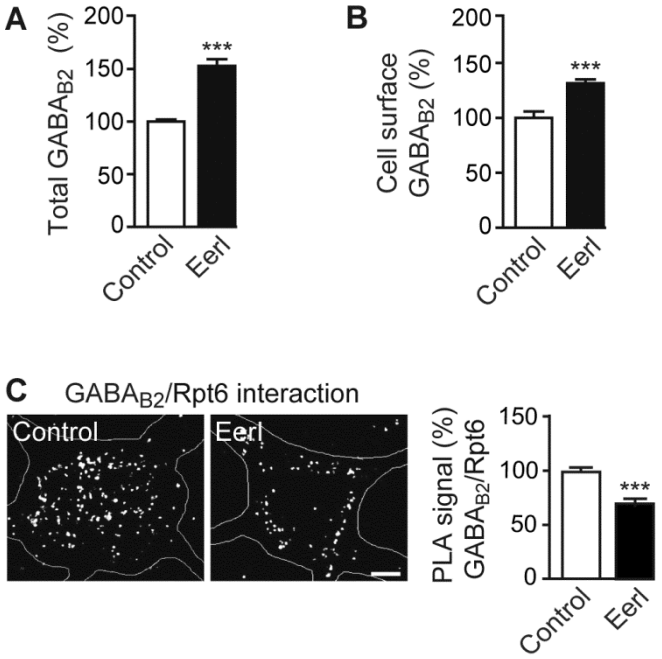

FIGURE 6. **Blocking ERAD reduced $GABA_{B2}$/Rpt6 interaction and increased $GABA_B$ receptor expression.**

A-B, blocking the ERAD pathway increased the level of $GABA_B$ receptors. Neurons grown in 96-well plates were incubated for 12 h with the ERAD inhibitor Eeyarestatin I (EerI, 5 μM). Total (*A*) and cell surface (*B*) $GABA_B$ receptor levels were determined using the whole cell ELISA and $GABA_{B2}$ antibodies. Control, cultures not treated with Eeyarestatin I. Means ± S.E.M., 30 cultures from three preparations. ***, $p<0.0001$; t-test.

C, diminished $GABA_{B2}$/Rpt6 interaction upon inhibition of ERAD. Neurons were incubated for 12 h with the ERAD inhibitor Eeyarestatin I (EerI, 5 μM) and analyzed for interaction of $GABA_{B2}$ with Rpt6 using *in situ* PLA. Left: Representative images depicting PLA signals (white dots, scale bar: 5 μm). Right: quantification of *in situ* PLA signals. Means ± S.E.M., 28 neurons from three preparations. ***, $p<0.0001$; t-test.

Neuronal activity modulates $GABA_B$ receptor expression and $GABA_{B2}$/Rpt6 interaction: Proteasomal activity has been reported to be regulated by the level of neuronal activity (Djakovic et al 2009). Therefore, we tested whether changes in neuronal activity affects the amount of $GABA_B$ receptor. We pharmacologically manipulated the activity of cultured neurons and determined their $GABA_B$ receptor protein levels using $GABA_{B2}$ antibodies and the whole cell ELISA. Treatment of neurons for 12 h with CNQX/D-AP5 in order to block excitatory synaptic transmission by inhibiting AMPA and NMDA receptors considerably increased total and cell surface $GABA_{B2}$ levels (total: 129±4%, cell surface: 135±4% of control, Fig. 7 *A, B*). In contrast, chronically blocking $GABA_A$ receptor activity with picrotoxin (PTX) to elevate neuronal activity decreased total as well as cell surface $GABA_{B2}$ levels (total: 72±7%, cell surface: 83±1% of control, Fig. 7 *C, D*). The decrease in cell surface $GABA_{B2}$ was prevented by cotreatment with the ERAD inhibitor EerI (97±1% of control, Fig. 7*D*). This finding suggests that neuronal activity regulates $GABA_B$ receptor expression levels via modulating proteasomal activity associated with the ERAD machinery. Under our test conditions, blocking AMPA and NMDA receptors reduced proteasomal activity to 62±6% of control neurons as determined with a luminogenic proteasome substrate (Fig. 7*E*). By contrast, blocking $GABA_A$ receptors with PTX increased protasomal activity to 169±11% of control neurons (Fig. 7*E*). These findings suggest that changes in neuronal excitation controls the expression level of $GABA_B$ receptors via proteasomal degradation.

If neuronal activity indeed regulates total and cell surface $GABA_B$ receptor expression via proteasomal degradation the interaction of $GABA_{B2}$ with Rpt6 should be concomitantly regulated. Therefore, we expected a reduced level of $GABA_B$ receptor/Rpt6 interaction after treating neuronal cultures for 12 h with CNQX plus D-AP5, conditions that leads to diminished proteasome activity. Indeed, blocking glutamate receptors considerably reduced the level of $GABA_{B2}$/Rpt6 interaction (39±4% of control, Fig. 7*F*). In contrast, enhancing neuronal activity by blocking $GABA_A$ receptors, which enhanced proteasome activity, increased the number of $GABA_{B2}$/Rpt6 interactions (302±22% of control, Fig. 7*F*). These findings suggest that neuronal activity modulates cell surface expression of $GABA_B$ receptors via changes in proteasomal activity, which is reflected by changes in the level of $GABA_{B2}$/Rpt6 interaction.

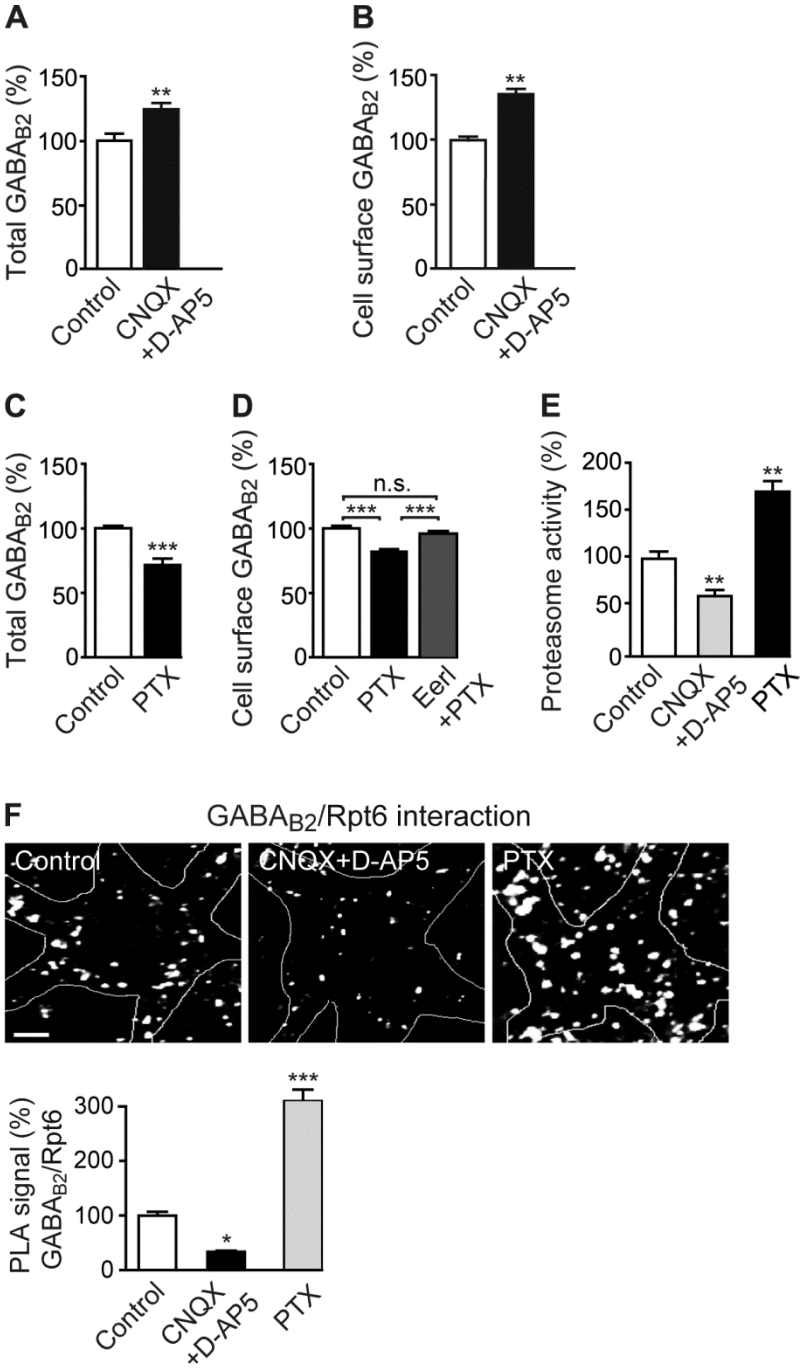

FIGURE 7. **The expression level of $GABA_B$ receptors and interaction with Rpt6 is controlled by neuronal activity.**

A-D, chronic changes in neuronal activity modulate $GABA_B$ receptor expression. Neurons grown in 96-well plates were incubated for 12 hours with 10 μM CNQX/20 μM D-AP5 (*A, B*) or with 20 μM picrotoxin (PTX, *C, D*) in the absence or presence of the ERAD inhibitor Eeyarestatin I (EerI, 5 μM) followed by determination of total (*A, C*) and cell surface (*B, D*) $GABA_{B2}$ levels using the whole cell ELISA. Means ± S.E.M., 20-47 cultures from 2-4 experiments. (*A, B, C*) **, $p<0.01$; ***$p<0.0001$; t-test; (*D*) ***$p<0.0001$, n.s. ($p>0.05$); one way ANOVA, Bonferroni's Multiple Comparison post-hoc test.

E, chronic changes in neuronal activity modulate proteasome activity. Proteasome activity was determined in neurons grown in 96-well plates incubated for 12 h with 10 μM CNQX/20 μM D-AP5

or with 20 μM picrotoxin (PTX) using the Proteasome-Glo cell-based assay. Means ± S.E.M., 12 cultures from four preparations. **, $p<0.01$, one way ANOVA, Dunnett's post-hoc test.
F, chronic changes in neuronal activity modulate interaction of $GABA_{B2}$ with Rpt6. Neurons were incubated for 12 hours with 10 μM CNQX/20 μM D-AP5 or with 20 μM picrotoxin (PTX) followed by determination of the $GABA_{B2}$/Rpt6 interaction levels using *in situ* PLA. Top: Representative images depicting the PLA signals (white dots, scale bar: 5 μm). Bottom: quantification of *in situ* PLA signals. Means ± S.E.M., 37 neurons from 2 experiments. *, $p<0.05$, ***, $p<0.0001$, one way ANOVA, Dunnetts post-hoc test.

DISCUSSION

$GABA_B$ receptors are degraded by two distinct pathways. Cell surface $GABA_B$ receptors are constitutively internalized via the dynamin and clathrin-dependent pathway, recycled to the plasma membrane and are eventually sorted to lysosomes for degradation (Benke 2010). Newly synthetized and assembled $GABA_B$ receptors in the ER are constitutively degraded to a certain extent by proteasomes via the ERAD machinery (Zemoura et al 2013a). This mechanism controls the pool of new $GABA_B$ receptors destined for forward trafficking to the plasma membrane. In the present study we show that the proteasomal AAA-ATPase Rpt6 interacts with the C-terminus of $GABA_{B2}$ thereby mediating proteasomal degradation of $GABA_B$ receptors. Rpt6 is one of the six AAA-ATPases of the 19S regulatory particle of the proteasome (Rubin et al 1996). The six distinct AAA-ATPases located at the base of the 19S regulatory particle of the 26S proteasome recognize, unfold, and translocate the protein substrates into the 20S, protein-degrading, core particle (Smith et al 2006, Sorokin et al 2009). Initially, a yeast-two hybrid screening with a peptide comprising the last 12 C-terminal amino acids of $GABA_{B2}$ suggested that Rpt6 may interact with $GABA_{B2}$. Co-immunoprecipitation from rat brain extracts, colocalization in cultured neurons and testing the association in neurons using *in situ* PLA verified the interaction of Rpt6 with $GABA_B$ receptors. This was further substantiated by experiments inhibiting proteasome acitivty which increased the interaction and colocalization of Rpt6 with $GABA_B$ receptors. Finally, the interaction of Rpt6 with the C-terminus of $GABA_{B2}$ was confirmed by peptide competition.

Experiments in HEK 293 cells support the view that Rpt6 is essential for proteasomal degradation of $GABA_B$ receptors. It had been previously reported that overexpression of Rpt6 in HEK 293 cells increase proteasome activity threefold, most likely due to a regulatory role of free Rpt6 on proteasome assembly and thereby activity (Wang et al 2012). In line with this report, overexpression of Rpt6 in HEK 293 cells downregulated total and cell surface $GABA_B$ receptors. Downregulation depended on the Rpt6/$GABA_{B2}$ interaction as well as on the Lys^{48}-

linked ubiquitination of $Lys^{767/771}$ in $GABA_{B2}$. Because $Lys^{767/771}$ ubiquitination in $GABA_{B2}$ is a prerequisite for proteasomal degradation of $GABA_B$ receptors (Zemoura et al 2013a) these findings underline that the interaction of $GABA_{B2}$ with Rpt6 is required for proteasomal degradation of the receptors.

Although Rpt6 does not interact with $GABA_{B1}$, as indicated by the lack of its downregulation when expressed in HEK 293 cells in the absence of $GABA_{B2}$, $GABA_{B1}$ and $GABA_{B2}$ were concomitantly reduced to the same extent. This strongly suggests that assembled $GABA_B$ receptor complexes are degraded by proteasomes and is in line with our previous observation that heterodimeric receptor complexes are degraded by the ERAD machinery and not single $GABA_B$ receptor subunits before being assembled in the ER (Zemoura et al 2013a). Our finding that blocking ERAD function increased total and cell surface $GABA_B$ expression and reduced the level of $GABA_{B2}$/Rpt6 interaction verifies that proteasomal degradation of $GABA_B$ receptors takes place at the ER.

Our data further indicate that proteasomal degradation of $GABA_B$ receptors in neurons require intact ATPase activity of Rpt6 because overexpression in neurons of a mutant Rpt6 lacking ATPase activity led to an increase of total as well as cell surface $GABA_B$ receptors. This finding provides further evidence that $GABA_B$ receptors are constitutively degraded to a certain extent by proteasomes (Zemoura et al 2013a). Lack of ATPase activity did not affect the interaction of Rpt6 with $GABA_{B2}$, suggesting that recruiting $GABA_B$ receptors to proteasomes is independent of Rpt6's ATPase activity.

Proteins targeted for proteasomal degradation are deubiquitinated before being degraded by the 20S proteasome. Lys48-linked polyubiquitin of substrate proteins is bound by Rpn10 and released by the deubiquitinase activity of Rpn11 present in the 19S regulatory particle (Sorokin et al 2009). Overexpression of mutant Rpt6 in neurons considerably reduced the fraction of Lys48-linked ubiquitinated $GABA_{B2}$ although the receptors were not degraded and remained bound to Rpt6. This suggests that deubiquitination of $GABA_{B2}$ is independent of its degradation and takes place before or concomitant with its translocation into the degradation chamber of the 20S proteasome.

There is accumulating evidence that neuronal activity regulates proteasome-dependent protein degradation and, intriguingly, proteasome activity. It has recently been demonstrated that blockade of neuronal activity decrease proteasomal activity whereas enhancing neuronal activity increase proteasomal activity (Djakovic et al 2012, Djakovic et al 2009, Jakawich et al 2010). The mechanism of enhancing proteasomal activity involves Ca^{2+} influx via NMDA receptors as well as L-type voltage-gated Ca^{2+} channels (Djakovic et al 2009). This leads to

the activation of CaMKII which phosphorylates Rpt6 on Ser^{120} (Bingol et al 2010, Djakovic et al 2012, Djakovic et al 2009) to roughly double proteasomal activity (Bingol et al 2010, Djakovic et al 2012). Conversely, reduced neuronal activity decreases phosphorylation of Rpt6 (Djakovic et al 2012) resulting in diminished proteasomal activity (Djakovic et al 2009, Jakawich et al 2010). In view of this data, it was not surprising that blocking neuronal activity reduced the level of $GABA_{B2}$/Rpt6 interaction and upregulated total as well as cell surface $GABA_B$ receptors. On the other hand, increasing neuronal activity by blocking $GABA_A$ receptors, which enhances proteasomal activity, increased the level $GABA_{B2}$/Rpt6 interaction and consequently decreased the expression levels of $GABA_B$ receptors. This was reversed by pharmacologically blocking ERAD demonstrating that proteasomal degradation affects the pool of newly synthetized $GABA_B$ receptors present in the ER. These findings are in line with the view that the level of neuronal activity regulates proteasomal activity and thereby the pool of $GABA_B$ receptors available in the ER for trafficking to the plasma membrane.

In conclusion, our data support the hypothesis that cell surface trafficking of $GABA_B$ receptors is controlled by neuronal activity at the level of the ER by defining the amount of receptors present in the ER via regulated proteasomal degradation. This mechanism is expected to contribute to homeostatic synaptic plasticity.

STUDY III:

CaMKII-dependent K63-linked ubiquitination of $GABA_{B1}$ drives lysosomal degradation of $GABA_B$ receptors

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Abstract

$GABA_B$ receptors are heterodimeric G protein-coupled receptors, which control neuronal excitability by mediating slow and prolonged inhibition. The magnitude of $GABA_B$ receptor-mediated inhibition essentially depends on the amount of receptors in the plasma membrane. One factor that determines receptor availability is regulated protein degradation. $GABA_B$ receptors are constitutively internalized and either recycled to the cell surface or degraded in lysosomes. The signal that sorts $GABA_B$ receptors to lysosomes is currently unknown. Here we tested whether ubiquitination is the lysosomal sorting signal for $GABA_B$ receptors. We found that inhibition of lysosomal activity in cortical neurons increased total and cell surface $GABA_B$ receptors as well as the level of K63-linked ubiquitinated receptors. Mutational inactivation of four putative ubiquitination sites in the $GABA_{B1}$ subunit significantly diminished K63-linked ubiquitination of $GABA_B$ receptors and prevented their lysosomal degradation. Searching for factors that control lysosomal degradation of $GABA_B$ receptors revealed that blocking Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) decreased K63-linked ubiquitination of $GABA_B$ receptors and inhibited lysosomal degradation of $GABA_B$ receptors by sorting the receptors to the recycling pathway. Finally, triggering lysosomal degradation of $GABA_B$ receptors by sustained activation of glutamate receptors, a condition mimicking in brain ischemia, was accompanied with a massive increase of CaMKII-dependent K63-linked ubiquitination of $GABA_B$ receptors. Preventing K63-linked ubiquitination by blocking CaMKII, overexpressing ubiquitin mutants or mutant $GABA_B$ receptors deficient in $GABA_{B1}$ K63-linked ubiquitination prevented down-regulation of the receptors. These findings indicate that CaMKII-dependent K63-linked ubiquitination of $GABA_{B1}$ at multiple sites controls sorting of $GABA_B$ receptors to lysosomes for degradation under physiological and pathological condition.

Introduction

$GABA_B$ receptors are heterodimeric G protein-coupled receptors assembled from $GABA_{B1}$ and $GABA_{B2}$ subunits. They are activated by γ-aminobutyric acid (GABA), the main inhibitory neurotransmitter in the brain. $GABA_B$ receptors are abundantly expressed in pre- and postsynaptic compartments of inhibitory as well as excitatory neurons to regulate their excitability. At presynaptic locations, $GABA_B$ receptors suppress neurotransmitter release mainly by inhibiting voltage-gated Ca^{2+} channels whereas at postsynaptic sites they induce slow inhibitory postsynaptic currents by activating Kir3-type K^+ channels (Benke et al 2012). $GABA_B$ receptors are involved in the regulation of all main brain functions ranging from synaptic plasticity (Pinard et al 2010), neuronal network activity (Craig & McBain 2014, Kohl & Paulsen 2010) to neuronal development (Gaiarsa & Porcher 2013). Accordingly, dysfunction of $GABA_B$ receptor signalling has been implicated in a variety of neurological disorders (Benarroch 2012, Bowery 2006, Cryan & Kaupmann 2005, Kumar et al 2013).

For understanding the contribution of $GABA_B$ receptors to physiological and pathological mechanisms the elucidation of its regulation is essential. A main factor regulating $GABA_B$ receptor signalling is the dynamic control of their cell surface expression. In this respect, protein degradation is one important mechanism that regulates the number of $GABA_B$ receptors at the cell surface. For instance, the amount of $GABA_B$ receptors in the endoplasmic reticulum (ER) available for forward trafficking to the cell surface is determined by the rate of their proteasomal degradation via the ERAD (ER associated degradation) machinery (Zemoura et al 2013). Proteasomal degradation of ER residing $GABA_B$ receptors is regulated by neuronal activity and requires K48-linked ubiquitination of $GABA_{B2}$ as well as its interaction with the proteasomal AAA-ATPase Rpt6 (Zemoura & Benke 2014, Zemoura et al 2013). On the other hand, $GABA_B$ receptors at the cell surface are constitutively endocytosed and either recycled to the cell surface or degraded in lysosomes (Grampp et al 2008, Grampp et al 2007, Hannan et al 2011, Kantamneni et al 2008, Vargas et al 2008). Lysosomal degradation of $GABA_B$ receptors is most likely mediated via the ESCRT (endosomal sorting complex required for transport) machinery (Kantamneni et al 2008), which sorts ubiquitinated membrane proteins to lysosomes (Raiborg & Stenmark 2009). Lysosomal degradation of $GABA_B$ receptors appears to be tightly regulated since excessive activity of glutamate receptors, a condition that occurs in brain ischemia, rapidly down-regulates $GABA_B$ receptors by preferential sorting the receptors to the lysosomal degradation pathway at the expense of receptor recycling (Guetg et al 2010, Kantamneni et al 2014, Maier et al 2010, Terunuma et al 2010).

The specific signal(s) that sorts $GABA_B$ receptors to lysosomal degradation under normal as well as pathological condition is currently unknown. Here we show that Ca^{2+}/calmodulin-dependent protein kinase (CaMKII) mediated K63-linked ubiquitination of $GABA_{B1}$ at multiple sites targets $GABA_B$ receptors to the lysosomal degradation pathway.

Materials and Methods

Antibodies. The following antibodies were used: Anti-HA mouse (1:1000 for immunofluorescence, 1:200 for *in situ* PLA, Sigma-Aldrich), rabbit $GABA_{B1b}$ directed against the N-terminus of $GABA_{B1b}$ (affinity-purified, 1:200 for immunofluorescence, custom made by GenScript) (Benke et al 1999), rabbit $GABA_{B2}$ directed against the N-terminus of $GABA_{B2}$ (affinity-purified, 1:500 for immunofluorescence; custom made by GenScript) (Benke et al 2002), guinea pig $GABA_{B2}$ (1:500 for immunofluorescence; Millipore), mouse $GABA_{B1}$ (1:100 for PLA; NeuroMab), rabbit ubiquitin K48-specific (clone Apu2, 1:50 for *in situ* PLA; Millipore), rabbit ubiquitin K63-specific (clone Apu3, 1:50 for *in situ* PLA; Millipore), goat Rab7 (1:250 for immunofluorescence; Santa Cruz Biotechnology), mouse Rab11 (1:40 for immunofluorescence; BD Transduction Laboratories). Secondary antibodies were labeled with either Alexa Fluor 488 (1:1000, Invitrogen), Cy-3 (1:500, Jackson ImmunoResearch Laboratories) or Cy-5 (1:300, Jackson ImmunoResearch Laboratories).

Drugs. The following chemicals were used for this study: glutamate (50 µM, Sigma-Aldrich), KN93 (10 µM, Tocris Bioscience) and leupeptin (100 µM, Sigma-Aldrich).

Plasmids. The following DNAs were used: $GABA_{B(1a)}$ (Kaupmann et al 1997); $GABA_{B2}$ (Kaupmann et al 1998); HA-tagged ubiquitin (Addgene plasmid 17608), HA-tagged ubiquitin (KO) (Addgene plasmid 17603), HA-tagged ubiquitin K63 (Addgene plasmid 17606) and HA-tagged ubiquitin K48R (Addgene plasmid 17604) (Lim et al 2005); HA-tagged ubiquitin K63R was kindly provided by L-Y Liu-Chen, Temple University, Philadelphia, USA; wild type EGFP-tagged rab7 (Addgene plasmid 12605) and the functionally inactive mutant EGFP-tagged rab7(DN) (Addgene plasmid 12660) (Choudhury et al 2002); wild type EGFP-tagged rab11 (Addgene plasmid 12674) and the a functionally inactive mutant EGFP-tagged rab11(DN) (Addgene plasmid 12678) (Choudhury et al 2002).

Mutation of $GABA_{B1}$. Lysines 697, 698, 892 and 960 in $GABA_{B1}$ were mutated to arginines using the QuikChange II XL site-directed mutagenesis kit from Stratagene according to the manufacturer's instructions.

Culture and transfection of cortical neurons. Primary neuronal cultures of cerebral cortex were prepared from 18 day old embryos of Wistar rats as described previously (Grampp et al 2008). Neurons were used after 11 to 15 days in culture. Plasmid DNA was transfected into neurons by magnetofection using Lipofectamine 2000 (Invitrogen) and CombiMag (OZ Biosciences) as detailed in (Buerli et al 2007).

Immunocytochemistry and confocal laser scanning microscopy. Immunofluorescence staining was performed as described previously (Grampp et al 2008, Maier et al 2014). For

selective detection of cell surface $GABA_B$ receptors living neurons were incubated with antibodies recognizing the extracellular located N-terminal domain of $GABA_{B1}$ or $GABA_{B2}$ for one hour at 4°C. For analysis of total $GABA_B$ receptors, neurons were fixed with 4% paraformaldehyde for 156-20 min at room temperature and permeabilized with 0.2% Triton X-100 before immunostaining.

Stained neurons were analyzed by laser scanning confocal microscopy (LSM 510 Meta, Zeiss). Images of eight optical sections spaced by 0.3 μm were recorded with a 100x plan-fluar oil differential interference contrast objective (1.45 NA, Zeiss) at a resolution of 1024 x 1024 pixels. Quantitative analysis of total and cell surface staining was performed as described in (Maier et al 2014).

Quantification of colocalization of $GABA_B$ receptors with Rab7 and Rab11 was done with the Mac Biophotonics Image J software (JACoP plugin, "Colocalization Threshold"). This method of quantitative colocalization analysis of fluorescence signals is based on an algorithm that automatically set the thresholds for colocalization using spatial statistics and thus excludes bias by the experimenter (Costes et al 2004).

In-cell Western assay. Total $GABA_B$ receptor expression of neurons cultured in 96-well plates was analyzed using the in-cell Western assay exactly as described previously (Maier et al 2010). Fluorescence signals generated by $GABA_{B1}$ and $GABA_{B2}$ antibodies were normalized to actin signals determined simultaneously in the same cultures.

In situ proximity ligation assay (in situ PLA). In situ PLA is an antibody based technology for the detection of protein-protein interactions and posttranslational modifications of proteins in cells *in situ* (Leuchowius et al 2010, Soderberg et al 2006). The *in situ* PLA was performed using Duolink PLA probes and detection reagents (Olink Bioscience, Sigma-Aldrich) according to the manufacturer's instructions as described previously (Maier et al 2014). Here we applied *in situ* PLA for the detection and quantification of $GABA_B$ receptor ubiquitination using mouse $GABA_{B1}$ or mouse HA antibodies together with rabbit antibodies specifically detecting K48-linked or K63-linked ubiquitin. Quantification was done by counting individual *in situ* PLA spots using the Mac Biophotonics Image J software. The number of spots was normalized to the area analyzed and to the expression level of $GABA_B$ receptors.

Statistics. The statistical analyses were done with GraphPad Prism 5. The tests used and *p* values are given in the figure legends. Differences were considered statistically significant when $p < 0.05$.

Results

Lysosomal degradation regulates cell surface expression of $GABA_B$ receptors

$GABA_B$ receptors undergo fast constitutive dynamin and clathrin-dependent endocytosis. Most of the receptors are recycled to the plasma membrane whereas a minor fraction is sorted to the lysosomal degradation pathway (Grampp et al 2008, Grampp et al 2007, Laffray et al 2007, Vargas et al 2008, Wilkins et al 2008). However, it is currently not known whether interfering with lysosomal degradation affects the expression of cell surface expression of $GABA_B$ receptors. Blocking lysosomal degradation in cultured cortical neurons with leupeptin for 12 hours considerably increased total ($GABA_{B1}$, 151.4 ± 6%; $GABA_{B2}$, 159.8 ± 7% of control, Fig. 1*A*) as well as cell surface expression of $GABA_B$ receptors ($GABA_{B1}$, 145.9 ± 9%; $GABA_{B2}$, 146.7 ± 9% of control Fig. 1*B*) to a similar extent. This suggests that constitutive lysosomal degradation is one factor determining the availability of $GABA_B$ receptors at the cell surface for signaling.

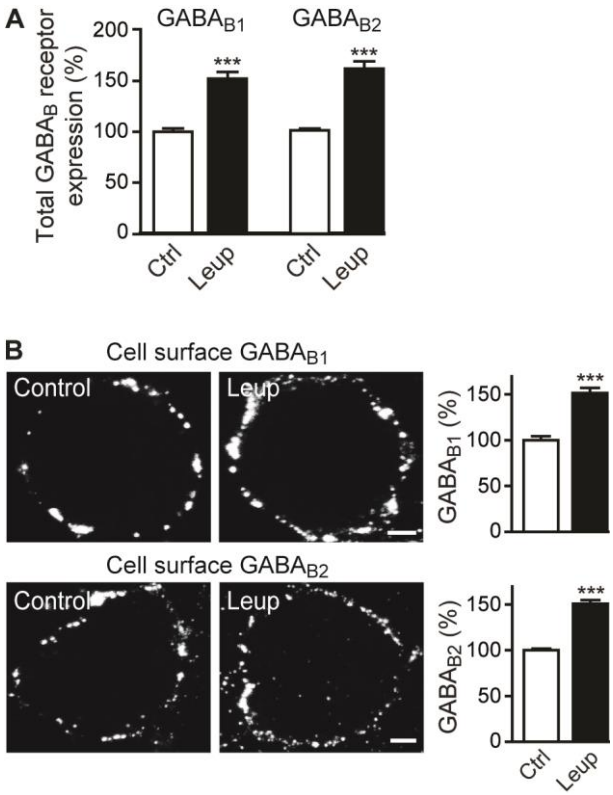

Figure 1. The expression level of $GABA_B$ receptors is controlled by lysosomes

A, The total expression level of $GABA_B$ receptors is increased in neurons after blocking lysosomal activity. Cortical neurons were incubated for 12 hours with 100 µM leupeptin followed by immunostaining for total $GABA_{B1}$ and $GABA_{B2}$ using the in-cell western technique. Neurons not treated with leupeptin served as controls. Fluorescence intensities for $GABA_{B1}$ and $GABA_{B2}$ in control neurons were set to 100%. The data represent the mean ± SEM of 30 cultures from three independent experiments. *** $p< 0.0001$; two-tailed unpaired t-test.

B, Expression of cell surface $GABA_B$ receptors is increased in neurons after inhibiting lysosomal activity. Cortical neurons were treated as indicated in ***A*** and immunostained for cell surface $GABA_{B1}$ and $GABA_{B2}$. Left, representative images of the soma of stained neurons. Scale bar, 5 µm. Right, the

graphs show the quantification of fluorescence intensities. Fluorescence intensities for $GABA_{B1}$ and $GABA_{B2}$ in control neurons were set to 100%. The data represent the mean ± SEM of 30-40 neurons from three independent experiments. *** p< 0.0002; two-tailed unpaired t-test.

K63-linked ubiquitination is involved in lysosomal degradation of $GABA_B$ receptors

K48-linked ubiquitination tags proteins for degradation in proteasomes whereas K63-linked ubiquitination is involved in non-proteolytic functions and can serve as a sorting signal for lysosomal degradation (Komander & Rape 2012). To test whether K63-linked ubiquitination is involved in lysosomal degradation of $GABA_B$ receptors neurons were transfected with a mutant of ubiquitin that is not able to form K63-linked chains (Ub(K63R)) and analyzed for cell surface expression of $GABA_B$ receptors. Inhibition of K63-linked ubiquitination by overexpression of Ub(K63R) increased the expression level of cell surface $GABA_B$ receptors (136±9% of control neurons transfected with wild type ubiquitin; Fig. 2*A*), suggesting that $GABA_B$ receptor levels are regulated by K63-linked ubiquitination.

Next we tested whether regulation of $GABA_B$ receptor levels by lysosomal degradation requires direct K63-linked ubiquitination of the receptor by *in situ* PLA using antibodies directed against $GABA_{B1}$ and K63-linked ubiquitin. Under basal conditions, $GABA_B$ receptors exhibited K63-linked ubiquitination, which was considerably increased upon inhibition of lysosomal activity with leupeptin (164 ± 8 % of control, Fig. 2*B*). In contrast, the level of $GABA_B$ receptor K48-linked ubiquitination, which target the receptors to proteasomal degradation (Zemoura & Benke 2014, Zemoura et al 2013), remained unaffected by blocking lysosomal activity (Fig. 2*C*). This suggests that direct K63-linked ubiquitination of $GABA_B$ receptors regulates lysosomal degradation of $GABA_B$ receptors.

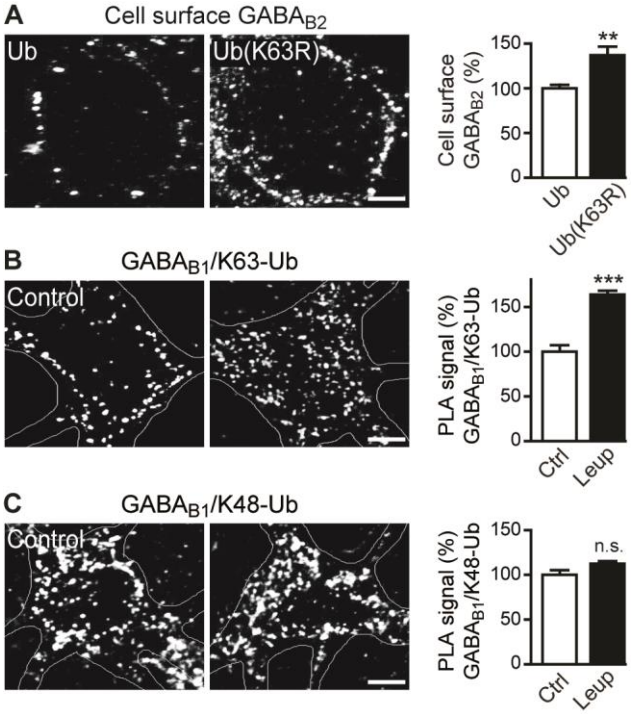

Figure 2. **The expression level of GABA$_B$ receptors is regulated by K63-linked ubiquitination.**
A, Interference with K63-linked ubiquitination increased the expression level of cell surface GABA$_B$ receptors. Neurons were transfected with wild type ubiquitin (Ub) or a ubiquitin mutant unable to form K63-linked chains (Ub(K63R)) and analyzed for GABA$_B$ receptor expression using GABA$_{B2}$ antibodies. Left, representative images of stained neuronal somata (scale bar, 5 μm). Right, quantification of fluorescence intensities. The fluorescence signal of neurons transfected with wild type ubiquitin was set to 100%. The data represent the mean ± SEM of 34 neurons from three independent experiments. ** $p < 0.004$; two-tailed unpaired t-test.
B, Inhibition of lysosomal activity enhanced K63-linked ubiquitination of GABA$_B$ receptors. Cortical neurons were incubated for 12 hour with or without (control) 100 μM leupeptin and analyzed for K63-linked ubiquitination by *in situ PLA* using antibodies directed against GABA$_{B1}$ and K63-linked ubiquitin (white dots in representative images, scale bar: 5 μm.). Right, quantification of *in situ* PLA signals. The data represent the mean ± SEM of 30-40 neurons from three independent experiments. *** $p < 0.00001$; two-tailed unpaired t-test.
C, Inhibition of lysosomal activity did not affect K48-linked ubiquitination of GABA$_B$ receptors. Cortical neurons were treated as in ***B*** and analyzed for K48-linked ubiquitination by *in situ PLA* using antibodies directed against GABA$_{B1}$ and K48-linked ubiquitin (white dots in representative images, scale bar: 5 μm.). Right, quantification of *in situ* PLA signals. The data represent the mean ± SEM of 27-37 neurons from three independent experiments; n.s., $p > 0.05$; two-tailed unpaired t-test.

Identification of K63-linked ubiquitination sites in GABA$_{B1}$

Because K63-linked ubiquitination appears to be involved in lysosomal degradation of GABA$_B$ receptors, we searched for potential lysine residues serving as ubiquitination sites by an *in silico* analysis of the GABA$_{B1}$ sequence. Four lysines with a high probability of being ubiquitinated were identified: two in the cytoplasmatic loop linking transmembrane domains three and four and two in the C terminal domain (Fig. 3*A*). These sites were inactivated by mutation to arginine (K->R), resulting in the three GABA$_{B1}$ mutants GABA$_{B1a}$(K697/698R), GABA$_{B1a}$(K892R) and GABA$_{B1a}$(K960R). To test whether these sites are ubiquitinated, cultured cortical neurons were transfected with either wild type GABA$_{B1a}$ or one of the GABA$_{B1a}$(K->R) mutants as well as GABA$_{B2}$ and analyzed for K63-linked

ubiquitination by *in situ* PLA. Numerous *in situ* PLA signals in neurons transfected with wild type $GABA_{B1a}$ indicated that $GABA_{B1}$ is K63-linked ubiquitinated under basal conditions. In contrast, all three mutant $GABA_{B1a}$ displayed strongly reduced K63-linked ubiquitination ($GABA_{B1a}$(K697/698R): 43 ± 3%, $GABA_{B1a}$(K892R): 38 ± 3%, $GABA_{B1a}$(K960R): 37 ±3%, of wild type $GABA_{B1a}$; Fig. 3*B*). This result indicates that lysines 697 and/or 698, lysine 983 and lysine 960 in $GABA_{B1}$ subunit are K63-linked ubiquitinated under basal conditions.

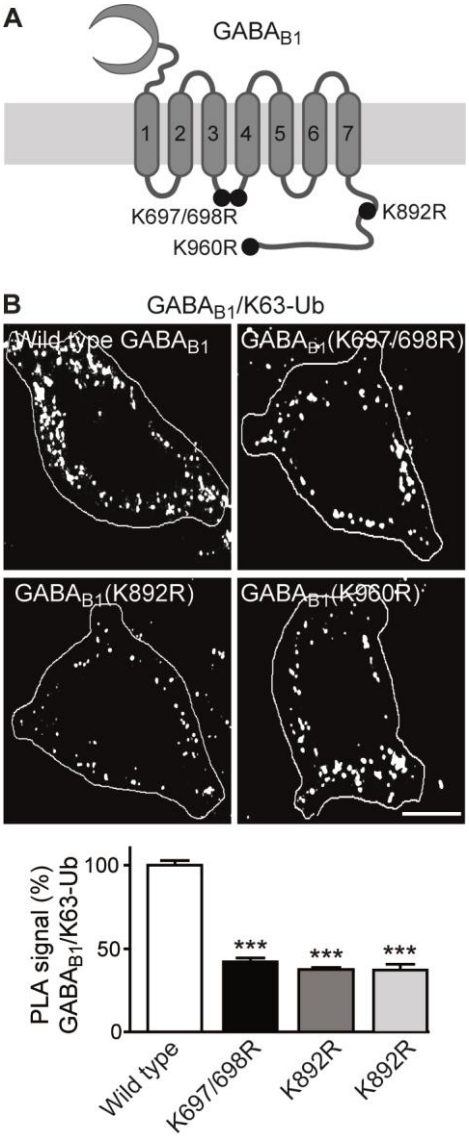

Figure 3. Identification of K63-linked ubiquitination sites in $GABA_{B1}$
A, Cartoon depicting the location of K->R mutations in $GABA_{B1}$.
B, Decreased K63-linked ubiquitination of $GABA_{B1}$(K->R) mutants. Cortical neurons were transfected with HA-tagged wildtype $GABA_{B1a}$, HA-tagged $GABA_{B1a}$(K697/698R), HA-tagged $GABA_{B1a}$(K892R) or HA-tagged $GABA_{B1a}$(K960R) together with wild type $GABA_{B2}$ and analyzed for K63-linked ubiquitination by *in situ* PLA using antibodies directed against the HA-tag and K63-linked ubiquitin (white dots in representative images, the dots in the outlined area were counted for quantification; scale bar: 7 µm). Right, quantification of *in situ* PLA signals. The data represent the mean ± SEM of 26-35 neurons from three independent experiments. *** p< 0.0001; one way ANOVA, Dunnett's Multiple Comparison

Ubiquitination of $GABA_{B1}$ regulates cell surface expression of $GABA_B$ receptors

To analyze the effect of K63-linked ubiquitination on cell surface expression of $GABA_B$ receptors, neurons were transfected either with wild type $GABA_{B1}$ or $GABA_{B1a}$(K->R) mutants along with $GABA_{B2}$ and immunostained for their total and cell surface expression levels. Total as well as cell surface expression of all three $GABA_{B1a}$ mutants was considerably increased as compared to transfected wildtype $GABA_{B1a}$ (total, $GABA_{B1a}$(K697/698R): 457 ± 26%, $GABA_{B1a}$(K892R): 511 ± 30%, $GABA_{B1a}$(K960R): 551 ± 22%, of wild type $GABA_{B1}$; cell surface: $GABA_{B1a}$(K697/698R): 508 ± 52%, $GABA_{B1a}$(K892R): 504 ± 48%, $GABA_{B1a}$(K960R): 482.2 ± 42% of wild type $GABA_{B1}$, Fig. 4*A, B*). Likewise, the cell surface expression of $GABA_{B2}$ in neurons transfected with $GABA_{B1a}$ mutants was significantly increased ($GABA_{B2}$ in $GABA_{B1a}$ (K697/698R) transfected neurons: 158 ± 14%, $GABA_{B2}$ in $GABA_{B1a}$(K892R) transfected neurons: 187 ± 17%, $GABA_{B2}$ in $GABA_{B1a}$(K960R) transfected neurons: 178 ±16% of control; Fig. 4*B*). The considerably lower increase in $GABA_{B2}$ cell surface expression as compared to mutant $GABA_{B1}$ was due to the fact that in case of $GABA_{B1}$ only transfected subunits were assayed (Ha-tagged) while in the case of $GABA_{B2}$ transfected as well as endogenously expressed subunits were detected. These results demonstrate that inactivation of any of the three ubiquitination sites in $GABA_{B1}$ (K697/698, K892, K960) decreased or prevented degradation of $GABA_B$ receptors and therefore increased their cell surface expression.

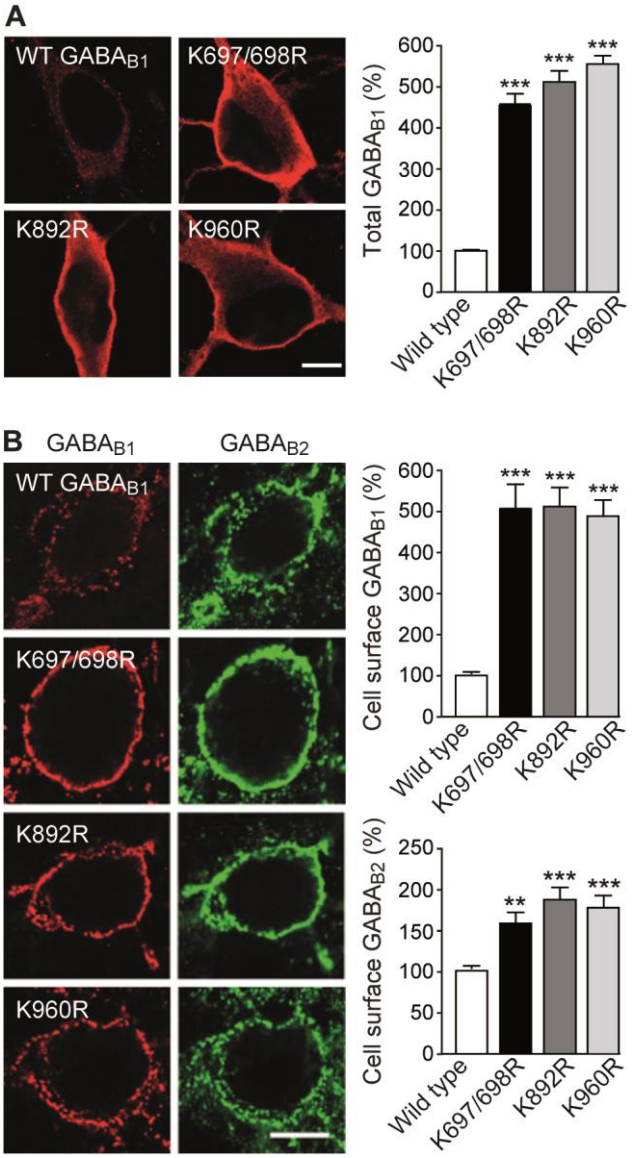

Figure 4. GABA$_{B1}$(K->R) mutants exhibit increased total and cell surface expression.
A, Increased total expression levels of GABA$_{B1a}$(K->R) mutants. Neurons were transfected with HA-tagged wild type GABA$_{B1a}$, HA-tagged GABA$_{B1a}$(K697/698R), HA-tagged GABA$_{B1a}$(K892R) or HA-tagged GABA$_{B1a}$(K960R) together with wild type GABA$_{B2}$ and analyzed for the expression level of transfected GABA$_{B1}$ using antibodies directed against the HA-tag. Left, representative images (scale bar: 7 µm). Right, quantification of fluorescence signals. The fluorescence signals of neurons transfected with wild type GABA$_{B1}$ was set to 100%. The Data represent the mean ± SEM of 23-27 neurons per experimental condition derived from three independent experiments. *** $p < 0.0001$; one way ANOVA, Dunnett's Multiple Comparison test.
B, Increased cell surface expression levels of GABA$_{B1a}$(K->R) mutants. Neurons were transfected with HA-tagged wild type GABA$_{B1a}$, HA-tagged GABA$_{B1a}$(K697/698R), HA-tagged GABA$_{B1a}$(K892R) or HA-tagged GABA$_{B1a}$(K960R) together with wild type GABA$_{B2}$ and analyzed for cell surface expression levels of transfected GABA$_{B1}$ as well as transfected plus endogenous GABA$_{B2}$ using antibodies directed against the HA-tag and GABA$_{B2}$, respectively. Left, representative images (scale bar: 7 µm). Right, quantification of fluorescence signals. The fluorescence signals of neurons transfected with wild type GABA$_{B1}$ or wild type GABA$_{B2}$, respectively, was set to 100%. The Data represent the mean ± SEM of 26-28 neurons per experimental condition derived from three independent experiments. ** $p < 0.001$; *** $p < 0.0001$; one way ANOVA, Dunnett's Multiple Comparison test.

Lysosomal targeting of $GABA_B$ receptors is regulated by ubiquitination of $GABA_{B1}$

The increased total and cell surface expression levels of $GABA_{B1a}$(K->R) mutants and their reduced K63-linked polyubiquitination suggests that ubiquitination of these lysine residues serve as signals for sorting the receptors to lysosomes for degradation. If this is the case, $GABA_{B1a}$(K->R) mutants should be resistant to lysosomal degradation and their expression levels should not increase upon blocking lysosomal degradation. Indeed, in contrast to the expression level of wild type $GABA_{B1}$, those of all three $GABA_{B1a}$(K->R) mutants remained unaffected by inhibition of lysosomal degradation with leupeptin (wild type $GABA_{B1}$: 249 ± 30%, $GABA_{B1a}$ (K697/698R): 111 ± 5%, $GABA_{B1a}$ (K892R): 109 ± 5%, $GABA_{B1a}$ (K960R): 108 ± 4% of control, Fig. 5*A*).

To confirm this finding, we prevented lysosomal degradation by overexpressing a functionally inactive mutant of the small GTPase Rab7 (Rab7(DN)). Rab7 mediates trafficking from early endosomes via late endosomes to lysosomes (Ng et al 2012) and therefore overexpression of Rab7(DN) disrupts this pathway. As expected, overexpression of Rab7(DN) considerably enhanced total expression of wild type $GABA_{B1a}$ but did not significantly affect the expression levels of $GABA_{B1a}$(K->R) mutants (wild type $GABA_{B1a}$: 174 ± 11%, $GABA_{B1a}$ (K697/698R): 105 ± 8%, $GABA_{B1a}$ (K892R): 117 ± 8%, $GABA_{B1a}$ (K960R): 126 ± 9% of control; Fig. 5*B*). This indicates that preventing ubiquitination of specific sites in $GABA_{B1}$ excluded the mutant receptors from entering the endosomal pathway that directs proteins to the lysosome. Therefore, our observations suggest that ubiquitination of multiple lysine residues in $GABA_{B1}$ receptors regulates lysosomal degradation of $GABA_B$ receptors.

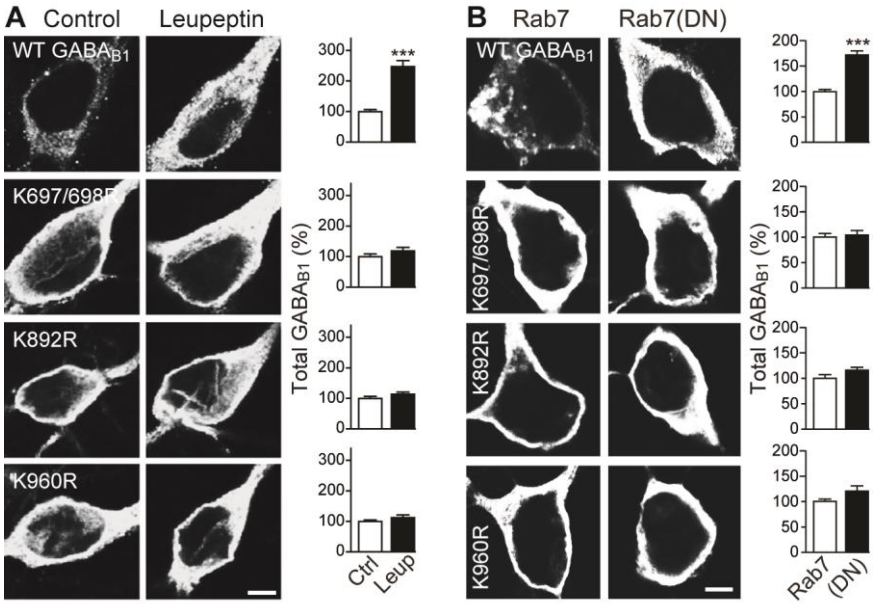

Figure 5. The expression levels of $GABA_{B1a}$(K->R) mutants are unaffected by inhibition of lysosomal degradation

A, Total expression levels of $GABA_{B1a}$(K->R) mutants are unaffected by blocking lysosomal activity with leupeptin. Neurons were transfected with HA-tagged wildtype $GABA_{B1a}$ or HA-tagged $GABA_{B1a}$(K->R) mutants together with $GABA_{B2}$, incubated with 100 µM leupeptin for 12 hours followed by immunostaining for transfected HA-tagged $GABA_{B1}$ using HA-antibodies. Left, representative images of untreated neurons (control, left) and of neurons incubated with leupeptin (right, scale bar: 7 µm). Right, quantification of fluorescence intensities. The fluorescence intensity of $GABA_{B1a}$ from untreated neurons (control) was set to 100%. The data represent the mean ± SEM of 27-34 neurons per experimental condition derived from three independent experiments. *** p< 0.0001, two-tailed unpaired t-test.

B, Total expression levels of $GABA_{B1a}$ (K->R) mutants are unaffected upon blocking lysosomal targeting by inactivation of Rab7. Neurons were transfected with HA-tagged wild type $GABA_{B1a}$ or $GABA_{B1a}$(K->R) mutants together with $GABA_{B2}$ and with either wild type Rab7 or with a non-funtional mutant of Rab7 (Rab7(DN)) and analyzed for total expression levels of transfected $GABA_{B1}$ using HA antibodies. Left, representative images depicting total expression of transfected $GABA_{B1a}$ (scale bar: 7 µm). Right, quantification of fluorescence intensities. The fluorescence intensities of $GABA_{B1a}$ coexpressed with wild type Rab7 were set to 100%. The data represent the mean ± SEM of 27-34 neurons derived from three independent experiments. *** p< 0.0001, two-tailed unpaired t-test.

CaMKII controls sorting of $GABA_B$ receptor to lysosomes

It was recently shown that CaMKII binds to $GABA_B$ receptors and phosphorylates $GABA_{B1}$ at serine 867 (Guetg et al 2010). CaMKII-mediated phosphorylation of $GABA_{B1}$ serine 867 plays a key role in glutamate receptor-induced down-regulation of $GABA_B$ receptors, a condition that mimics ischemia (Guetg et al 2010). We therefore analyzed the involvement of CaMKII in the regulation of $GABA_B$ receptor cell surface expression under basal conditions using the CaMKII inhibitor KN93. Inhibition of CaMKII by KN93 increased the level of $GABA_B$ receptors at the cell surface (148 ± 10% of control; Fig. 6*A*), suggesting that basal CaMKII activity restricts cell surface expression of the receptors.

To get an insight into the mechanism that is affected by CaMKII inhibition, we tested the co-localization of $GABA_B$ receptors with Rab7 (marker for late endosomes and lysosomes) and Rab11 (marker for recycling endosomes) (Ng et al 2012). Inhibition of CaMKII activity in neurons with KN93 decreased the number of $GABA_B$ receptor clusters co-localizing with Rab7 (80 ± 3% of control; Fig. 6*B*) and increased the number of $GABA_B$ receptor clusters co-localizing with Rab11 (139 ± 9%, of control; Fig. 6*C*). Thus, inhibition of CaMKII appears to prevent recruitment of $GABA_B$ receptors to the lysosomal degradation pathway and enhances recycling of the receptors to the plasma membrane. This observation suggests that basal CaMKII activity is involved in targeting of $GABA_B$ receptors to lysosomes for degradation.

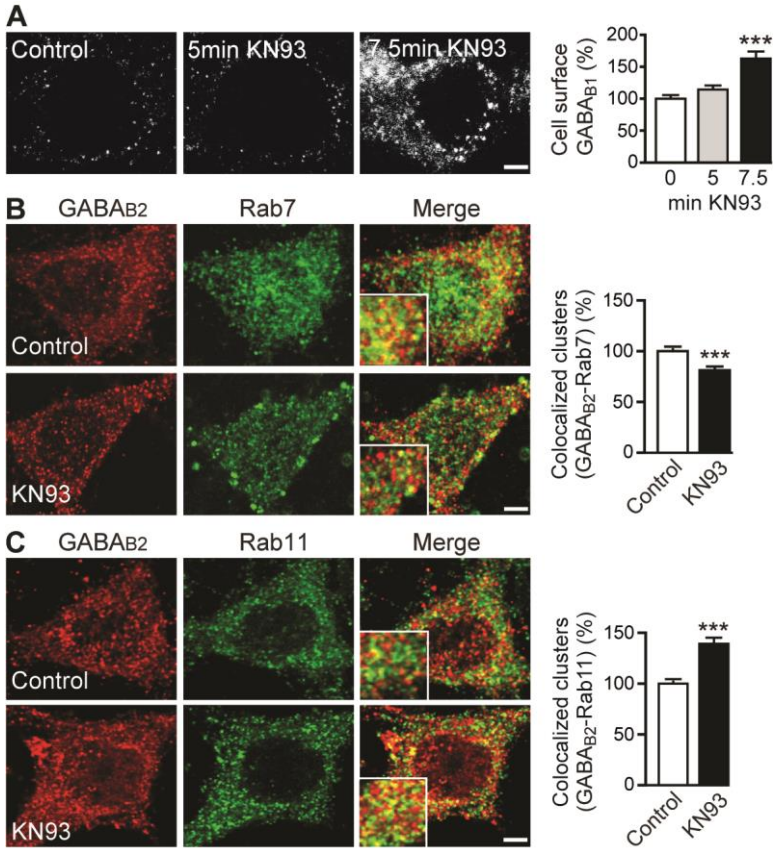

Figure 6. CaMKII regulates lysosomal degradation of $GABA_B$ receptors.
A, Inhibition of CaMKII activity increased cell surface expression of $GABA_B$ receptors. Neurons were incubated for the indicated times with the CaMKII inhibitor KN93 (10 μM) followed by determination of cell surface $GABA_B$ receptors expression using $GABA_{B1}$ antibodies. The immunofluorescence signals of untreated control neurons were set to 100%. Left, representative images (scale bar: 5 μm). Right, quantification of fluorescence intensities. The data represent the mean ± SEM of 39-43 neurons from three independent experiments. *** $p< 0.0001$; one way ANOVA, Dunnett's Multiple Comparison test.
B, ***C***, Blocking CaMKII activity decreased the colocalization of $GABA_B$ receptors with the Rab7 (marker for the lysosomal pathway) and increased the colocalization with Rab11 (marker for recycling endosomes). Neurons were treated with KN93 and immunostained for $GABA_{B2}$ (red) and Rab7 or Rab11 (green). The yellow clusters in the merged image indicate the co-localization of $GABA_{B2}$ with Rab7 or Rab11, respectively (left, representative images; scale bars: 5 μm). Right, quantification of colocalization. The data represent the mean ± SEM of 30-60 neurons from three independent experiments; *** $p< 0.0005$, two-tailed unpaired t-test.

CaMKII promotes K63-linked ubiquitination of $GABA_B$ receptors

The results so far support the hypothesis that CaMKII regulates lysosomal degradation of $GABA_B$ receptors most likely by promoting K63-linked ubiquitination of $GABA_B$ receptors. To test this hypothesis, we first tested whether blocking CaMKII with KN93 affects K63-linked ubiquitination of $GABA_B$ receptors. Indeed, $GABA_B$ receptors in KN63-treated neurons were considerably less K63-linked ubiquitinated as compared to receptors in untreated neurons (69 ± 8% of control, Fig. 7*A*). This finding shows that CaMKII regulates K63-linked ubiquitination of $GABA_B$ receptors.

Next; we tested whether blocking CaMKII affects cell surface expression of the three $GABA_{B1a}$(K->R) mutants, which are partially resistant to K63-linked ubiquitination. In contrast wild type $GABA_{B1}$, which displayed increased cell surface expression upon treatment with KN63 ($GABA_{B1}$: 164 ± 20%, $GABA_{B2}$: 155 ± 17% of control; Fig. 7*B*), the expression level of any of the three $GABA_{B1a}$(K->R) mutants remained unaffected by inhibition of CaMKII ($GABA_{B1a}$(K697/698R): 86 ± 5%, $GABA_{B1a}$ (K892R): 91 ± 6%, $GABA_{B1a}$ (K960R): 99 ± 9% of control, $GABA_{B2}$ co-expressed with $GABA_{B1a}$ (K697/698R), 85 ± 6%, $GABA_{B2}$ co-expressed with $GABA_{B1a}$(K892R): 92 ± 7%, $GABA_{B2}$ co-expressed with $GABA_{B1a}$(K960R): 100 ±7% of control; Fig. 7*B*). In conclusion, these findings suggest that CaMKII promotes K63-linked ubiquitination of $GABA_{B1}$ at K697/698, K892 as well as K960, which targets $GABA_B$ receptors to lysosomes for degradation.

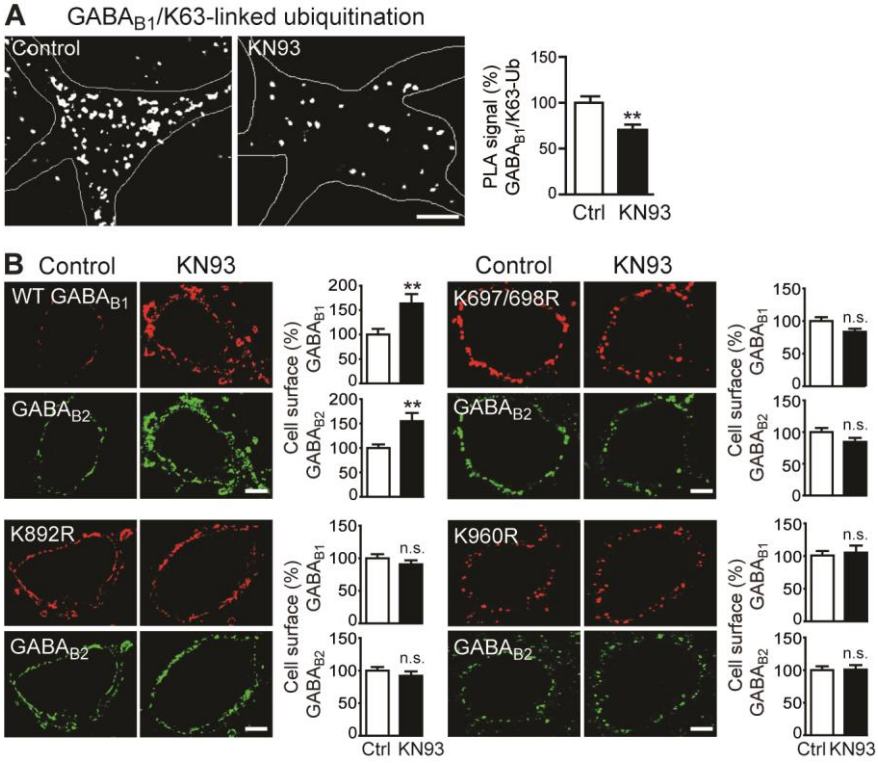

Figure 7. CaMKII regulates Lys63-linked polyubiquitination of GABA$_B$ receptors.
A, Inhibition of CaMKII activity reduced K63-linked ubiquitination of GABA$_B$ receptors. Neurons were incubated for 7.5 minutes in the absence (control) or presence of KN93 and analyzed for K63-linked ubiquitination of GABAB receptor by *in situ* PLA using antibodies directed against GABA$_{B1}$ and K63-linked ubiquitin (white dots in representative images, left panel, scale bar: 5 µm). Right, quantification of *in situ* PLA signals. The data represent the mean ± SEM of 33 neurons from two independent experiments. **, p< 0.0013, two-tailed unpaired t-test.
B, Blocking CaMKII did not affect the expression levels of GABA$_{B1a}$(K->R) mutants. Neurons were transfected with HA-tagged wild type GABA$_{B1a}$ or HA-tagged GABA$_{B1a}$ (K->R) mutants along with GABA$_{B2}$ and tested for cell surface expression of GABA$_{B1}$ and GABA$_{B2}$ after treating the neurons with 10 µM KN93 for 7.5 minutes. Left, representative images of untreated neurons (control, left panels) and of neurons treated with KN93 (right panels, scale bar: 5 µm). The corresponding graphs show the quantification of fluorescence signals. The fluorescence intensity of GABA$_{B1}$ or GABA$_{B2}$ from untreated neurons (control) was set to 100%. The data represent the mean ± SEM of 50-60 neurons per experimental condition derived from three independent experiments. ** p< 0.005, two-tailed unpaired t-test.

Sustained activation of glutamate receptors increases K63-linked ubiquitination of GABA$_B$ receptors

Sustained activation of glutamate receptors (AMPA as well as NMDA receptors) leads to the down-regulation of GABA$_B$ receptors via lysosomal degradation (Guetg et al 2010, Maier et al 2010, Terunuma et al 2010). To investigate whether K63-linked ubiquitination of GABA$_B$ receptors serves as a lysosomal sorting signal in this process, we exposed cortical neurons for 30 min to glutamate and tested for K63-linked ubiquitination using *in situ* PLA. Sustained activation of glutamate receptors dramatically increased K63-linked ubiquitination of GABA$_B$ receptors (524 ± 77% of control, Fig. 8*A*), which was completely prevented by blocking CaMKII with KN93 (123 ± 8% of control, Fig. 8*A*).

Next we tested whether preventing K63-linked ubiquitination inhibits the down-regulation of $GABA_B$ receptors after treating neurons with glutamate. For this, cortical neurons were transfected either with wild-type ubiquitin (Ub), a mutant of ubiquitin in which all lysines were mutated to arginines thereby preventing chain elongation and thus any kind polyubiquitination (Ub(KO)) or with a mutant in which all lysines were mutated to arginines except for K63 (Ub(K63), able to form only K63-linked ubiquitination) and stained for cell surface $GABA_B$ receptors after sustained glutamate application. Glutamate induced down-regulation of $GABA_B$ receptors from the plasma membrane in neurons expressing wild type ubiquitin (Ub(WT), 53 ± 5% of control, Fig. 8*B*), or the mutant that only permits K63-linked ubiquitination (Ub(K63), 61 ± 6% of control, Fig. 8*B*) but not in neurons expressing the mutant unable to build polyubiquitin chains (Ub(KO), 95 ± 11% of control, Fig. 8*B*).

To further confirm that ubiquitination of $GABA_{B1}$ is required for glutamate-induced down-regulation of $GABA_B$ receptors, we tested whether the expression of the $GABA_{B1a}$(K->R) mutants is affected by glutamate. However, in contrast to the cell surface expression of wild type $GABA_{B1}$ (56 ± 8% of control, Fig. 9), the levels of all three $GABA_{B1a}$(K->R) mutants remained unaffected by glutamate ($GABA_{B1a}$(K697/698R): 90 ± 12%, $GABA_{B1a}$(K892R): 115 ± 11%, $GABA_{B1a}$ (K960R): 108 ± 5% of control, Fig. 9).

These findings suggest that sustained activation of glutamate receptors induces CaMKII-mediated K63-linked ubiquitination of $GABA_B$ receptors, promoting their lysosomal degradation.

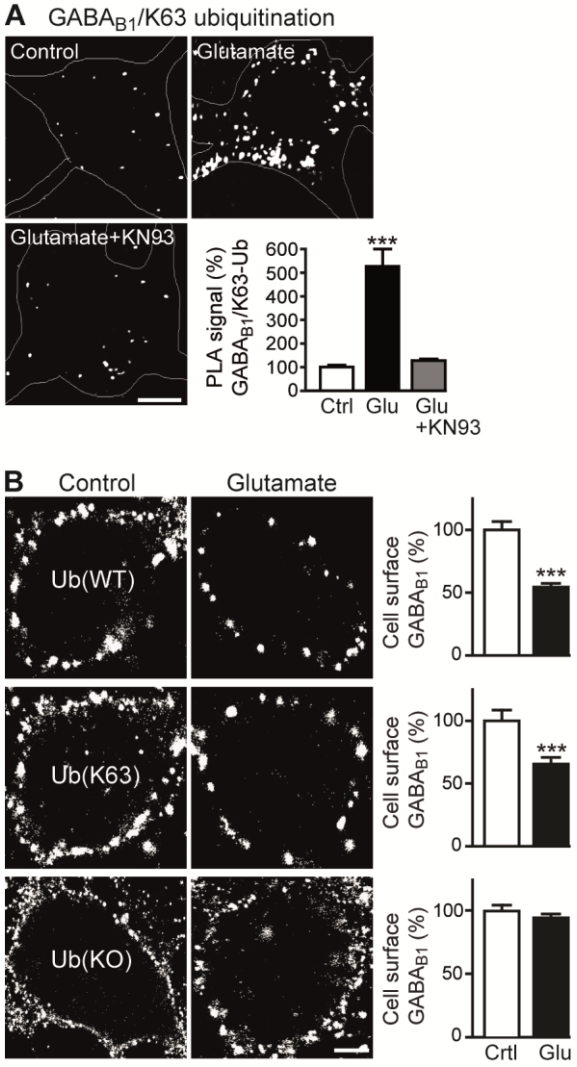

Figure 8. Glutamate-induced down-regulation of $GABA_B$ receptors is mediated by Lys63-linked polyubiquitination.

A, Sustained activation of glutamate receptors enhanced K63-linked ubiquitination of $GABA_B$ receptors. Neurons were incubated for 60 min in the absence (control) or presence of 50 μM glutamate with or without 10 μM KN93 and analyzed for K63-linked ubiquitination by *in situ* PLA using antibodies directed against $GABA_{B1}$ and K63-linked ubiquitin (white dots in representative images, scale bar: 5 μm). The graph depicts quantification of the *in situ* PLA signals. The data represent the mean ± SEM of 47-52 neurons derived from three independent experiments. *** $p < 0.0001$; one way ANOVA, Dunnett's Multiple Comparison test.

B, Preventing K63-linked ubiquitination rendered $GABA_B$ receptors resistant to glutamate-induced down-regulation. Neurons were transfected with wild-type ubiquitin (Ub(WT)), and mutants of ubiquitin that either permits only K63 ubiquitination (Ub(K63)) or prevents any kind of ubiquitin chain generation (Ub(KO)). Neurons were incubated for 90 min in the absence (control) or presence of 50 μM glutamate followed by determination of cell surface $GABA_B$ receptors using $GABA_{B1}$ antibodies. Left, representative images, scale bar: 5 μm. Right, quantification of fluorescence intensities. The data represent the mean ± SEM of 30-36 neurons from three independent experiments. *** $p < 0.0001$; two-tailed unpaired t-test.

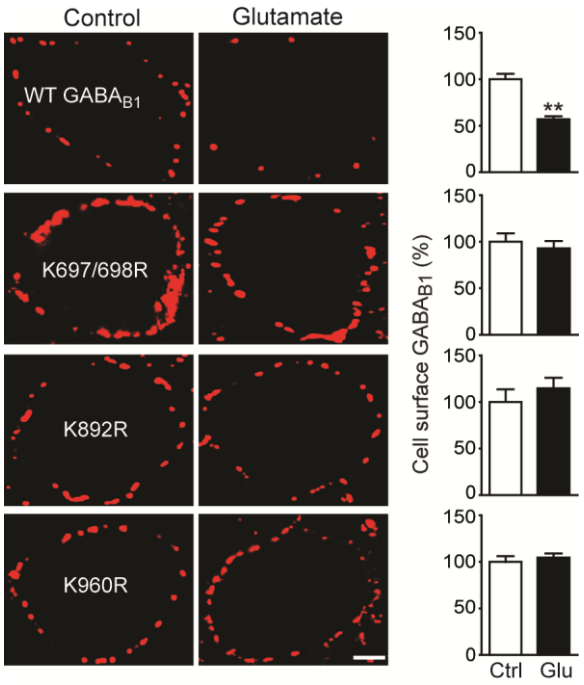

Figure 9. GABA$_{B1a}$(K->R) mutants are resistant to glutamate-induced down-regulation.
Neurons transfected with HA-tagged wildtype GABA$_{B1a}$ or HA-tagged GABA$_{B1a}$(K->R) mutants along with GABA$_{B2}$ were incubated in the presence (Glutamate) or absence (Control) of 50 µM glutamate for 90 minutes followed by cell surface staining for transfected GABA$_{B1}$ using HA antibodies. Left, representative images, scale bar: 5 µm. Right, quantification of fluorescence intensities. The fluorescence intensity of neurons not treated with glutamate was set to 100%. The data represent the mean ± SEM of 20-25 neurons per experimental condition derived from two independent experiments. *** p< 0.0005, two-tailed unpaired t-test.

Discussion

The signaling strength of G protein coupled receptors largely depends on the number of receptors present in the plasma membrane. The mechanisms determining cell surface expression of the receptors include exocytosis, endocytosis, recycling and degradation. $GABA_B$ receptors assemble into heterodimeric $GABA_{B1,2}$ complexes in the ER, which is a prerequisite for their ER exit and forward trafficking to the plasma membrane. After reaching the cell surface, $GABA_B$ receptors are constitutively internalized and either recycled to the plasma membrane or degraded in lysosomes (for a review see (Benke 2010)). Both, forward trafficking of $GABA_B$ receptors to the cell surface as well as their residence time at the cell surface are tightly regulated by controlled degradation of the receptors. The amount of $GABA_B$ receptors available for forward trafficking to the plasma membrane in the ER is adjusted by proteasomal degradation of the receptors via the ERAD machinery depending on the activity level of the neuron (Zemoura & Benke 2014, Zemoura et al 2013). On the other hand, the amount of receptors degraded in lysosomes after internalization from the cell surface depends on mechanisms sorting the endocytosed receptors to either lysosomes or recycling endosomes. Interfering with recycling rapidly depletes the receptors from the cell surface by redirecting them to the lysosomal degradation pathway (Grampp et al 2008). Rapid down-regulation of cell surface $GABA_B$ receptors by rerouting the receptors to lysosomes appears to be associated with pathological conditions as it is induced by sustained activation of glutamate receptors, which is a characteristic of brain ischemia (Guetg et al 2010, Kantamneni et al 2014, Maier et al 2010, Terunuma et al 2010). The factors triggering lysosomal degradation of $GABA_B$ receptors were however unknown. The results of the present study provided evidence that CaMKII-dependent K63-linked ubiquitination of $GABA_{B1}$ sorts $GABA_B$ receptors to lysosomes for degradation under physiological and pathological conditions.

We found that pharmacological inhibition of lysosomal activity increased not only total $GABA_B$ receptor levels, which was expected due to the intracellular accumulation of the receptors (Grampp et al 2007), but also considerably the enhanced cell surface expression of the receptors. This finding implies that regulating lysosomal degradation of $GABA_B$ receptors directly affects their cell surface expression, which in turn determines the strength of $GABA_B$ receptors signaling (Zemoura et al 2013). There is evidence that K63-linked ubiquitination is required for lysosomal degradation of $GABA_B$ receptors. First, blocking global K63-linked ubiquitination by overexpressing an ubiquitin mutant (K63R) that is unable to form K63-linked chains significantly increased cell surface expression of $GABA_B$ receptors. Second,

blocking lysosomal activity considerably increased the level of K63-linked ubiquitination of $GABA_B$ receptors while leaving the level of K48-linked ubiquitination, which tags the receptors for proteasomal degradation (Zemoura et al 2013), unaffected. Third, mutational inactivation of potential ubiquitination sites in $GABA_{B1}$ (K697/698, K892 and K960) strongly decreased K63-linked ubiquitination of $GABA_B$ receptors containing the respective $GABA_{B1}$ mutant and prevented their lysosomal degradation as indicated by their dramatically increased expression level and insensitivity to the effect of blocking lysosomal degradation (either by inhibiting lysosomal proteases by leupeptin or by overexpression of a functionally inactive mutant of Rab 7, which inhibits transport of cargo from late endosomes to the lysosome and blocks lysosome biogenesis). Any of the three $GABA_{B1}$ mutants (K697/698R, K892R and K960R) appeared to completely prevent lysosomal degradation of the receptors, suggesting that ubiquitination of K697/698, K892 and K960 in $GABA_{B1}$ is mandatory for lysosomal degradation of $GABA_B$ receptors. However, it is currently unclear at which stage of intracellular sorting these sites needs to be ubiquitinated. They may be ubiquitinated simultaneously at a certain sorting step or, alternatively, they may be sequentially ubiquitinated at distinct sorting checkpoints.

Lysosomal degradation of G protein coupled receptors is predominantly mediated via the ESCRT machinery (Dores & Trejo 2014), which guides mono- and K63-linked ubiquitinated membrane proteins to lysosomes (Lauwers et al 2009). Therefore, our observation that K63-linked ubiquitination tags $GABA_B$ receptors for lysosomal degradation indicates that the ESCRT machinery also sorts $GABA_B$ receptors to lysosomes. This view is supported by the finding that the ESCRT I complex component TGS101 (Hurley & Emr 2006) is required for lysosomal degradation of $GABA_B$ receptors (Kantamneni et al 2008).

Phosphorylation often regulates ubiquitination of proteins thereby promoting their degradation (Lin et al 2002, Schroeder et al 2012, Su et al 2013). Here we found that blocking CaMKII activity decreased K63-linked ubiquitination of $GABA_B$ receptors and increased their cell surface expression. Consistent with these findings, cell surface expression of all three $GABA_{B1a}$(K->R) mutants with inactivated K63-linked ubiquitination sites remained unaffected by CaMKII inhibition. These findings indicate that basal CaMKII activity determines the level of K63-linked ubiquitination of $GABA_B$ receptors and thus the extent of their lysosomal degradation. Our colocalization experiments suggests that blocking CaMKII impairs lysosomal sorting of the receptors (reduced colocalization with Rab7) thereby fostering receptor recycling (increased colocalization with Rab11). This redirection of $GABA_B$ receptors to the recycling pathway explains the increased cell surface expression of

receptors after interfering with lysosomal degradation. It is currently unclear whether direct phosphorylation of $GABA_B$ receptors is required or the phosphorylation of proteins involved in sorting of the receptors. However, the observation that lysosomal degradation triggered by sustained activation of glutamate receptors depends on phosphorylation of $GABA_{B1}$ by CaMKII (Guetg et al 2010) favors a direct phosphorylation of $GABA_{B1}$ by CaMKII as a prerequisite for its K63-linked ubiquitination.

Sustained activation of glutamate receptors, a situation occurring in brain ischemia, rapidly down-regulates $GABA_B$ receptors by increasing the rate of their lysosomal degradation at the expense of recycling (Guetg et al 2010, Kantamneni et al 2014, Maier et al 2010, Terunuma et al 2010). By overexpressing mutants of ubiquitin and the $GABA_{B1}$(K->R) mutants in neurons, we now provide evidence that glutamate-induced down-regulation of $GABA_B$ receptors depends on CaMKII-mediated K63-linked ubiquitination of $GABA_{B1}$. It was previously shown that glutamate-induced down-regulation of $GABA_B$ receptors critically depends on phosphorylation of $GABA_{B1}$ at serine 867 by CaMKII (Guetg et al 2010). This suggests that the massive Ca^{2+} influx induced by prolonged neuronal excitation over-activates CaMKII, resulting in excessive phosphorylation and consequently K63-linked ubiquitination of $GABA_{B1}$. In line with this view we observed a massive increase in K63-linked ubiquitination of $GABA_B$ receptors after sustained stimulation of glutamate receptors, which was completely abolished by blocking CaMKII.

In conclusion, our data suggest that CaMKII regulates K63-linked ubiquitination of $GABA_{B1}$ at four distinct sites, which sorts $GABA_B$ receptors to lysosomes for degradation. This mechanism is expected to fine-tune cell surface expression of $GABA_B$ receptors under physiological conditions and to considerably affect receptor expression in diseases associated with disturbed Ca^{2+} homeostasis.

General Discussion

Starting situation and research questions

$GABA_B$ receptors are important mediators in the modulation of neuronal function as they are responsible for slow and prolonged inhibitory transmission. Their signaling strength largely depends on the number of receptors present in the plasma membrane, which in turn is determined by their rate of exocytosis, endocytosis, recycling and degradation. In this thesis, we aimed at understanding if and how degradation pathways contribute to the regulation of cell surface expression of $GABA_B$ receptors. At the time of starting this research project several studies investigated how $GABA_B$ receptors reach the cell surface and a major mechanism for their endocytosis had been disclosed: the two subunits $GABA_{B1}$ and $GABA_{B2}$ exit the ER after their heterodimerisation, reach the cell surface and are then constitutively endocytosed via the clathrin- and dynamin-dependent pathway (Grampp et al 2008a, Grampp et al 2007b, Hannan et al 2011b, Margeta-Mitrovic et al 2000, Ramirez et al 2009, Vargas et al 2008). Once endocytosed, the majority of receptors is recycled to the plasma membrane, while a minority is degraded (Grampp et al 2008a, Grampp et al 2007b, Vargas et al 2008). In contrast, little was known about the routes and the regulation of $GABA_B$ receptor degradation. There was solid evidence for their degradation in lysosomes as $GABA_B$ receptors had been shown to colocalize with lysosomal marker proteins (Grampp et al 2008a, Hannan et al 2011a) and inhibition of lysosomal proteases led to their intracellular accumulation (Grampp et al., 2008; Maier et al., 2010). There was also indirect indication for ubiquitination being involved in lysosomal degradation of the receptors. Blocking global ubiquitination by inhibiting the ubiquitin-activating enzyme (E1) decreased lysosome-dependent glutamate-induced degradation of $GABA_B$ receptors (Maier et al., 2010). In addition, it had been shown that a subcomplex of the endosomal complex required for sorting (ESCRT), known to target ubiquitinated membrane proteins to lysosomes (Katzmann et al 2001), contributes to the degradation of $GABA_B$ receptors (Kantamneni et al., 2008). There were also hints that $GABA_B$ receptors might be degraded by proteasomes as a second route. Inhibition of proteasomes increased the expression level of $GABA_B$ receptors ectopically expressed in HEK293 cells (Kantamneni et al., 2008). Taken together, this background raised a number of basic questions that we aimed to address in this research project:

1. Are $GABA_B$ receptors degraded by proteasomes?
2. Is ubiquitination involved in proteasomal as well as lysosomal of $GABA_B$ receptors?
3. Where are relevant ubquitination sites located in $GABA_B$ receptors?
4. Does degradation regulate cell surface expression of $GABA_B$ receptors?
5. Is degradation of $GABA_B$ receptors regulated by neuronal activity?

In this thesis, we discovered that ubiquitin-mediated proteasomal as well as lysosomal degradation control cell surface expression of $GABA_B$ receptors depending on the activity level of the neurons.

Neuronal activity controls cell surface expression of $GABA_B$ receptors via ERAD-mediated proteasomal degradation

In this project we delineated a new mechanisms involved in the degradation of $GABA_B$ receptors. We found that $GABA_B$ receptors are K48-linked ubiquitinated at lysine residues 767/771 in the C-terminal domain of $GABA_{B2}$, which tags the receptors for proteasomal degradation (Zemoura et al 2013b). Proteasomal degradation involved the interaction of the C-terminus of $GABA_{B2}$ with the proteasomal AAA-ATPase Rpt6 (Zemoura & Benke 2014). We revealed that proteasomal degradation of $GABA_B$ receptors takes place at the ER, involves the ERAD machinery (Zemoura et al 2013b) and is regulated by neuronal activity (Zemoura & Benke 2014). Most importantly, we found that increasing or decreasing proteasomal degradation of $GABA_B$ receptors affects cell surface expression of the receptors. Our findings are consistent with a model in which neuronal activity controls the number of $GABA_B$ receptors present in the ER by proteasomal degradation via the ERAD pathway. This determines the number of receptors available for forward trafficking to the cell surface (Fig. 8).

A key role in the proteasomal degradation of $GABA_B$ receptors plays the interaction of the C-terminus of $GABA_{B2}$ with Rpt6, which is one of the six AAA-ATPases present in the 19S regulatory particle of the proteasome (Rubin et al 1996). The 19S regulatory particle of the proteasome is thought to recognize, unfold, and translocate the K48-linked polyubiquitinated protein substrates into the 20S, protein-degrading, core particle (Voges et al 1999). Rpt6 has been shown to interact with different proteins and is thought to control the recognition and thus degradation of proteins by the proteasome (Ferrell et al 2000). The transcription factor Sp1 as well as the estrogen receptors ER_a and ER_b are examples for proteins that undergo proteasomal degradation via interaction with Rpt6 (Han & Kudlow 1997, Masuyama & Hiramatsu 2004, Nawaz et al 1999). Our finding that blocking proteasomal activity increases the colocalization of $GABA_B$ receptors with Rpt6 and the number of $GABA_B$ receptor clusters in the dendrites of neurons as well as the observation that overexpression of a dominant-negative mutant of Rpt6 in neurons upregulates cell surface $GABA_B$ receptors demonstrates that Rpt6 mediates proteasomal degradation of $GABA_B$ receptors.

It is well established that misfolded and misassembled membrane proteins are rapidly removed from the ER membrane by the ERAD machinery and degraded in proteasomes located in the cytosol (Raiborg and Stenmark, 2009). Because $GABA_B$ receptors are transmembrane proteins that need to be removed from the membrane for proteasomal degradation, ERAD was the most likely pathway involved. Indeed, inhibition of proteasomal activity resulted in the accumulation of $GABA_B$ receptors in the ER and specific inhibition of the ERAD pathway by pharmacological tools or using a dominant-negative mutant of the AAA-ATPase p97, a component of the ERAD machinery, increased both total and cell surface $GABA_B$ receptor levels. These findings demonstrate that the ERAD pathway not only eliminates damaged membrane proteins but also regulates the number of receptors available for ER exit and transport to the cell surface. This view is supported by recent findings suggesting that ERAD is involved in the regulation of the abundance of synaptic $GABA_A$ receptors (Saliba et al., 2007).

Most importantly, we found that chronic pharmacological manipulation of neuronal activity alters cell surface expression of $GABA_B$ receptors via changes in the level of their ERAD-dependent proteasomal degradation (Zemoura & Benke 2014). Enhancement of neuronal activity reduced $GABA_B$ receptor cell surface expression whereas blocking neuronal activity increased their expression. Our observation that chronic changes in neuronal activity affect the activity of proteasomes is confirmed by published data (Djakovic et al., 2009; Jakawich et al., 2010; Djakovic et al., 2012) and provides - in part - a mechanistic link to the modulation of cell surface $GABA_B$ receptors. It has been shown that enhanced neuronal activity increases proteasome activity by a mechanism involving Ca^{2+} influx through NMDA receptors and L-type voltage-gated Ca^{2+} channels (Djakovic et al., 2009). Increased proteasomal activity down-regulates $GABA_B$ receptors in the ER and consequently limits their cell surface delivery. However, further experiments are required to address the question what mechanism controls the extent of K48-linked ubiquitination of $GABA_B$ receptors. This most likely involves neuronal activity-dependent phosphorylation events, which may regulate the activity of a specific E3 ubiquitin ligases and deubiqutinating enzymes (Huntwork-Rodriguez et al 2013) or primes the receptors for ubiquitination (Schwarz et al 2010).

What are the physiological implications of this regulatory mechanism? It has been shown that chronic suppression or elevation of activity in cultured neuronal networks exerts opposing compensatory effects on the excitatory glutamatergic and inhibitory GABAergic system. Blocking neuronal activity increases the amplitude of AMPA miniature excitatory postsynaptic currents (mEPSCs), correlating with enhanced levels of cell surface AMPA

receptors, whereas it decreases the amplitude of GABA miniature inhibitory postsynaptic currents (mIPSCs), corresponding to diminished $GABA_A$ receptor levels in the plasma membrane (Turrigiano 2011). This homeostatic response increases excitability of neurons and is one mechanism that contributes to stabilizing the firing rate of neurons within a range required for proper network function. The increased level of cell surface $GABA_B$ receptors after sustained suppression of neuronal activity may be required for tightly controlling the upregulated excitability of the neuron. In this sense, increased levels of $GABA_B$ receptors likely represent an emergency break to prevent overshooting excitation under conditions of enhanced neuronal activity.

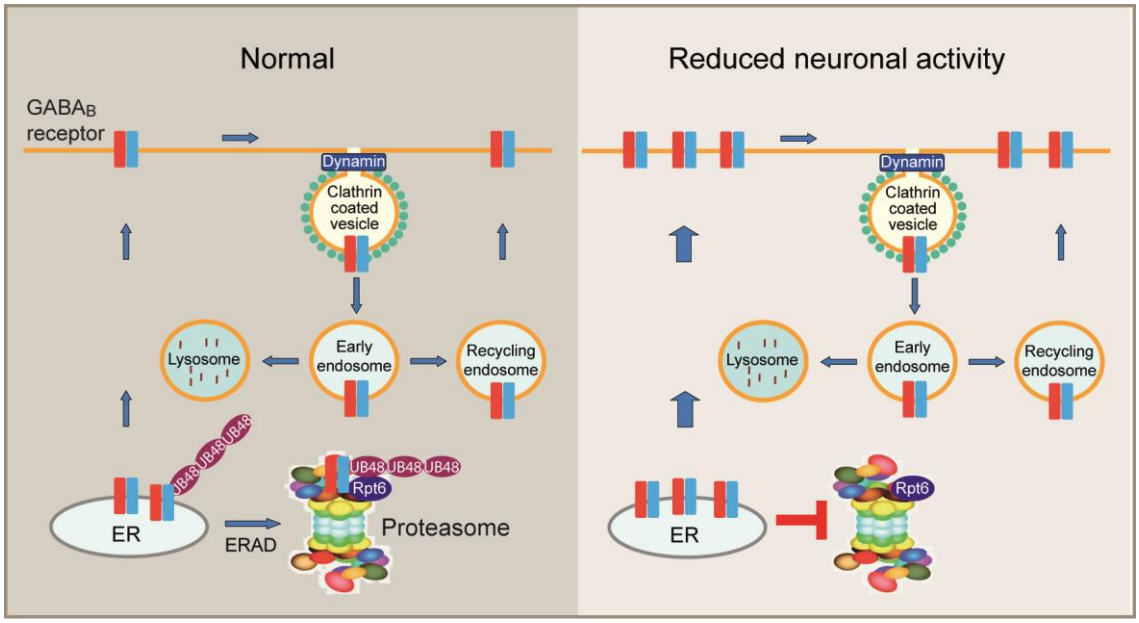

Figure 8: Proteasomal degradation pathway of $GABA_B$ receptors. $GABA_B$ receptors are synthetized at the ER and trafficked to the cell surface were they are constitutively internalized via the clathrin and dynamin-dependent pathway. The majority of endocytosed receptors are recycled to the plasma membrane, whereas a minority is degraded by lysosomes. The balance of all these events determines the amount of receptors on the cell surface and thus the maximum possible magnitude of signaling. Newly synthetized $GABA_B$ receptors in the ER are degraded by the ERAD machinery depending on the activity state of the neuron. This regulates the number of $GABA_B$ receptors in the ER available for forward trafficking to the plasma membrane and thus affects the level of cell surface receptors. Chronic depression of neuronal activity inhibits K48-linked polyubiquitination of $GABA_{B2}$ and their ERAD-mediated proteasomal degradation. Degradation of $GABA_B$ receptors by proteasomes requires K48-linked ubiquitination of $GABA_{B2}$ at lysines 761/771 and the interaction of its C-terminus with the proteasomal AAA-ATPase Rpt6.

CaMKII-dependent K63-linked ubiquitination and lysosomal degradation of $GABA_B$ receptors

In the second part of this thesis, we explored the role of lysosomal degradation in regulating the surface availability of $GABA_B$ receptors in neurons. We found that blocking lysosomal degradation indeed increased the expression level of cell surface $GABA_B$ receptors, which was dependent on K63-linked ubiquitination of $GABA_{B1}$ at four distinct lysine residues. We showed that blocking CaMKII activity decreased K63-linked ubiquitination of $GABA_{B1}$ and consequently inhibited lysosomal degradation of $GABA_B$ receptors. These results are consistent with a model in which CaMKII-mediated phosphorylation events (most likely direct phosphorylation of $GABA_{B1}$ at serine 867 (Guetg et al. 2010)) induces K63-linked ubiquitination of $GABA_{B1}$, which sorts the internalized receptors to lysosomes for degradation instead to the recycling pathway (Fig. 9). Our data imply that basal CaMKII activity under normal neuronal activity is sufficient to sort a minor fraction of $GABA_B$ receptors to lysosomes. Therefore, we hypothesize that CaMKII is part of a molecular switch that regulates the level of lysosomal degradation and recycling of $GABA_B$ receptors according to the activity state of the neuron. In line with this view, we found that sustained activation of glutamate receptors, which leads to a huge increase of intracellular Ca^{2+} levels and consequently enhanced CaMKII activity, induces massive CaMKII-mediated K63-linked ubiquitination of $GABA_B$ receptors and their down-regulation via lysosomal degradation (Fig. 9).

There are hints that this mechanism may regulate $GABA_B$ receptor expression *in vivo*. CaMKII has been implicated in both long term synaptic potentiation (LTP) as well as in long term synaptic depression (LTD), depending on the Ca^{2+} concentration, its isoform assembly (ratio of α and β isoforms) and autophosphorylation status (Coultrap & Bayer 2014, Pi et al 2010, van Woerden et al 2009). There is evidence that also $GABA_B$ receptors regulate LTD. Activation $GABA_B$ receptors in the induction phase has been shown to increase the magnitude of LTD by enhancing mGluR1-mediated Ca^{2+} release from intracellular stores (Kamikubo et al 2007). Interestingly, rats sleep deprived for 12 hours show enhanced LTD in the CA1 region of the hippocampus, which is associated with the upregulation of $GABA_B$ receptors (Tadavarty et al 2011). Considering that sleep deprivation decreases the expression level of CaMKII (Lopez et al 2008, Zagaar et al 2013, Zhang et al 2013), it may well be that the upregulation of $GABA_B$ receptors is due to reduced lysosomal degradation of $GABA_B$ receptors by diminished CaMKII-mediated K63-linked ubiquitination of $GABA_{B1}$.

Cerebral ischemia is another condition which is most likely associated with CaMKII-mediated regulation of $GABA_B$ receptor degradation. Ischemic stroke leads to a massive release of glutamate, resulting in neuronal overexcitation and eventually neuronal death. Under normal conditions, glutamatergic neurotransmission is tightly controlled by $GABA_B$ receptors to prevent excessive excitation (Chalifoux & Carter 2011b)**.** This mechanism is apparently not operative under ischemic conditions probably due to the down-regulation of $GABA_B$ receptors (Cimarosti et al 2009, Vollenweider et al 2006)**.**

Studies on cultured neurons mimicking the aspect of excessive glutamate receptor activation in cerebral ischemic revealed a rapid down-regulation of $GABA_B$ receptors, which was due to the re-routing of the receptors from the recycling pathway to lysosomal degradation and involved CaMKII-dependent phosphorylation of $GABA_{B1}$ (Guetg et al, 2010; Kantamneni et al, 2014; Maier et al, 2010; Terunuma et al, 2010). This mechanism was later confirmed in the oxygen and glucose deprivation (OGD) *in vitro* model of cerebral ischemia (Kantamneni et al, 2014). The results of this thesis uncovered the role of CaMKII in this mechanism. Glutamate-induced enhanced CaMKII activity promotes K63-linked ubiquitination of $GABA_{B1}$ and sorts the receptors, which normally recycle to the plasma membrane, to lysosomes for degradation. In the context of cerebral ischemia, the down-regulation of $GABA_B$ receptors is a detrimental event that is expected to foster excitotoxicity.

We hypothesize that interfering with this mechanism may provide an opportunity to restore normal $GABA_B$ receptor expression in order to increase the inhibitory control under ischemic conditions. This is expected to combat overexcitation and to limit excitotoxic neuronal death. Because $GABA_B$ receptors directly interact with CaMKII (Guetg et al. 2010) small synthetic peptides mimicking the CaMKII binding site can be employed for disrupting the CaMKII/$GABA_B$ receptor interaction. This is expected to prevent the CaMKII-mediated K63-linked ubiquitination of $GABA_{B1}$ and the down-regulation of the receptors. It needs to be tested if restoring normal $GABA_B$ receptor expression under ischemic conditions indeed limits neuronal death. If this is the case, this strategy may serve as a starting point for a highly specific therapeutic intervention based on cell permeable interfering peptides.

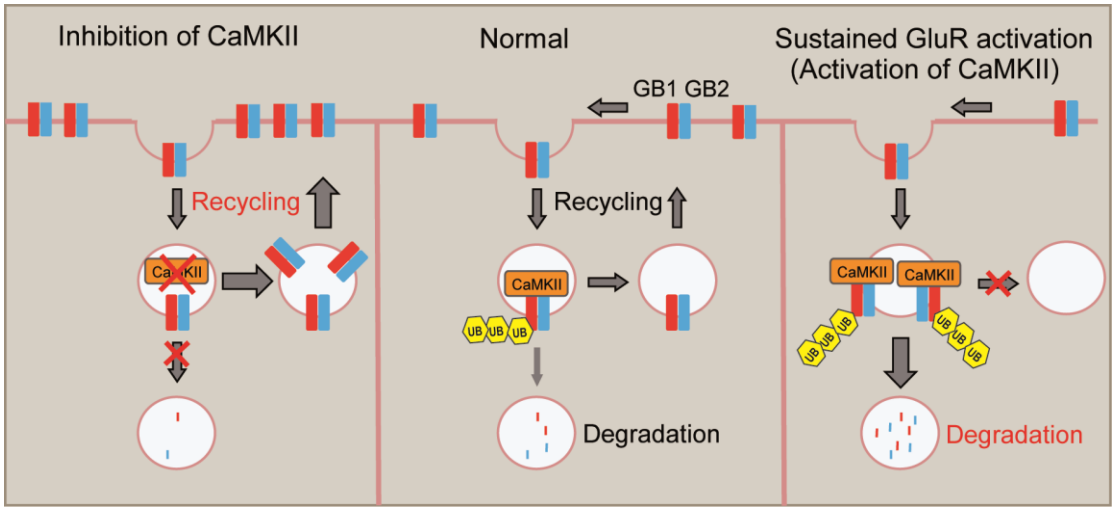

Figure 9: Lysosomal degradation pathway of $GABA_B$ receptors. Under normal conditions (middle), $GABA_B$ receptors are constitutively endocytosed from the plasma membrane and are sorted from early endosomes either to recycling endosomes or to lysosomes for degradation. CaMKII appears to play an important role in sorting the receptors to the lysosomal degradation pathway. CaMKII phosphorylation events (most likely the direct phosphorylation of $GABA_{B1}$) lead to K63-linked ubiquitination of $GABA_{B1}$, which serves as a signal for lysosomal targeting. The level of CaMKII activity determines the amount of receptors sorted to lysosomes and thereby regulates the amount of cell surface receptors. For instance, blocking CaMKII activity (left) inhibits K63-linked ubiquitination and lysosmal degradation of the receptors, which results in enhanced recycling and increased expression of cell surface receptors. On the contrary, overexcitation of neurons by sustained activation of glutamate receptors (right, GluR) enhances CaMKII activity, K63-linked ubiquitination of $GABA_{B1}$ and down-regulation of $GABA_B$ receptors. GB1: $GABA_{B1}$, GB2: $GABA_{B2}$, GluR: glutamate receptor, Ub, ubiquitin.

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ABBREVIATIONS

AAA-ATPase: ATPases associated with diverse cellular Activities

AMPA: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

AP2-complex: Adaptor protein 2 complex

CaMKII: Ca2+/calmodulin-dependent protein kinase II

cAMP: Cyclic adenosine monophosphate

CNQX: 6-cyano-7-nitroquinoxaline-2,3-dione

CNS: Central nervous system

COPI: coat protein complex I

DMEM: Dulbecco's modified eagle medium

E18: Embryonic of 18 days

ER: Endoplasmic reticulum

ER_a: Estrogen receptor a

ERAD: Endoplasmic-reticulum-associated protein degradation

ER_b: Estrogen receptor b

ESCRT: Endosomal sorting complex required for transport

GABA: Gamma-aminobutyric acid

$GABA_A$: Gamma-aminobutyric acid A receptor

$GABA_{B1a}$: Gamma-aminobutyric acid B receptor 1 type a

$GABA_{B1b}$: Gamma-aminobutyric acid B receptor 1 type b

$GABA_{B2}$: Gamma-aminobutyric acid B receptor 2

GPCR: G protein-coupled receptor

HEK 293: Human embryonic kidney 293 cells

HRD1: ERAD-associated E3 ubiquitin-protein ligase

KN-93: N-[2-[N-(4-Chlorocinnamyl)-N-methylaminomethyl]phenyl]-N-(2-hydroxyethyl)-4 methoxybenzenesulfonamide

mEPSC: miniature excitory postsynaptic current

mEPSCs: miniature excitatory postsynaptic currents

MG 132: N-[(Phenylmethoxy)carbonyl]-L-leucyl-N-[(1S)-1-formyl-3 -methylbutyl]-Lleucinamide

mIPSCs: miniature inhibitory postsynaptic currents

NMDA: N-methyl-D-aspartate

PBS: Phosphate-buffered saline

PLA: proximity ligation assay

PTX: Picrotoxin

Rab: Ras superfamily of monomeric G proteins

Rpt6: One of the six AAA-ATPase of the proteasomal 19S regulatory complex

Sp1: Stimulatory protein 1

sPSC :spontaneous postsynaptic current

TTX: Tétrodotoxine

Ub: ubiquitin

VCP (P97): Valosin-containing protein

WT: wild type

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